AD_____

Award Number: DAMD17-02-1-0579

TITLE: Novel COX-2 Inhibitor for Breast Cancer Therapy

PRINCIPAL INVESTIGATOR: E. Premkumar Reddy, Ph.D.

CONTRACTING ORGANIZATION: Temple University of the Commonwealth System of Higher Education – School of Medicine
Philadelphia, PA 19140

REPORT DATE: July 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-07-2005	Final	1 Jul 2002 – 30 Jun 2005

4. TITLE AND SUBTITLE
Novel COX-2 Inhibitor for Breast Cancer Therapy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-02-1-0579

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
E. Premkumar Reddy, Ph.D.

E-mail: reddy@temple.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Temple University of the Commonwealth System of Higher Education –
School of Medicine
Philadelphia, PA 19140

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Recent studies have shown that NSAIDS reduce the incidence of human cancers by inhibiting COX enzymes. Of these, the COX-2 isoform has been shown to be constitutively overexpressed in many tumor types, including those of the breast. The purpose of this study was to develop novel COX-2 inhibitors that can be used in breast cancer therapy. We have developed several classes of novel COX-2 inhibitors that inhibit the growth of both COX-2 positive and negative tumor cell lines, suggesting that they may target other protein(s) that play an important role in tumor cell proliferation. We have also determined that our most potent COX-2 inhibitor, which is nearly 6-fold more active than celecoxib, induced irreversible G1 arrest of tumor cells and ultimately led to tumor cell apoptosis. We have also determined the mechanism of action of these COX-2 inhibitors, which suggest an important role for these compounds as anti-cancer agents.

15. SUBJECT TERMS
COX-2, Inhibitors, Tumor Growth

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	26	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Cover……1

SF 298……..……2

Introduction…….……....4

Body……4.

Key Research Accomplishments…….……14

Reportable Outcomes……14

Conclusions……..15

References……15

Appendices……18

INTRODUCTION

Cyclooxygenase-1 and 2 (COX-1 and COX-2) catalyze the formation of prostaglandin H2 by converting arachadonic acid to prostaglandins (PGs) (Vane and Botting, 1998). Recent studies have shown that high levels of COX-2 are expressed in a large percentage of tumors, including those of the breast. In particular, constitutive over-expression of COX-2 has been observed in greater than 50% of ductal carcinomas in situ and in several highly metastatic estrogen receptor-negative breast tumor cell lines (Soslow et al, 2000; Liu and Rose, 1996). Furthermore, epidemiological studies have shown that the use of non-steroidal anti-inflammatory drugs (NSAIDs) can lower the incidence of certain tumor types, including those of the breast, and studies in animals have confirmed these findings (Prescott et al, 1996; Taketo, 1998; Williams et al, 1999; Badawi et al, 1998). Taken together, these studies provide compelling evidence to support the involvement of COX-2 in the development of breast cancer. It is therefore reasonable to conclude that drugs which target COX-2 enzymatic activity can have a profound impact on the treatment of this disease.

Celecoxib, a selective COX-2 inhibitor, has been shown to inhibit 7,12-dimethylbenz (a)anthracene (DMBA)-induced development of mammary tumors leading to their regression in animal model systems (Badawi et al, 1998, Alshafie et al, 2000). Because these studies demonstrate that COX-2 specific NSAIDs can act as both anti-carcinogenic and anti-neoplastic agents with respect to breast cancer, and because these types of NSAIDs are devoid of side effects, there is a need to develop new and improved agents to treat this disease.

BODY

Synthesis of novel COX-2 NSAIDs: To achieve the first aim of the proposal, we have synthesized additional series of compounds aimed at identifying the most potent COX-2 inhibitor that can be used in breast cancer therapy. These compounds belong to three classes: (i) the 18000 series compounds are additional derivatives of SKU-46 and include 8 compounds (18200, 18210, 18220, 18230, 18160, 18170, 18180 and 18190); (ii) the 53000 series of compounds are derivatives with a hydrazone backbone and include 20 compounds (53010, 53020, 53030, 53040, 53050, 53060, 53070, 53090, 53100, 53110, 53120, 53130, 53140, 53150, 53160,53210, 53220, 53230 and 53240); (iii) the 9000 series which belong to the pyrazoline class and include approximately 20 different compounds. Of these three series of compounds, the 18000 and 9000 series of compounds exhibited the most potent anti-COX-2 activity and hence were used for additional studies.

Studies with 18000 Series Compounds

Effect of the 18000 series compounds against COX-2 enzymatic activity: The inhibitory effect (reported as the IC_{50} value) of these drugs against the COX-2 (ovine) enzyme was analyzed using a COX inhibitory screening assay kit as described by the manufacturer (Cayman Chemicals, MI) (Gierse et al, 1999). This assay directly measures

the production of $PGE_{2\alpha}$, which is produced by stannous chloride reduction of COX-derived $PGEH_2$ via an enzyme immunoassay. This type of assay has been demonstrated to be more reliable than peroxide inhibition-based assay systems (Gierse et al, 1999). Celecoxib, which has an IC_{50} of 1.71μM was used as a control in all assays. The results of these studies show that three of the 18000 series of compounds (18100, 18010 and 18030) showed appreciable COX-2 inhibitory activity (Table 1). The methods used for the synthesis of these compounds and their relative inhibitory activities have been recently published by us (Reddy et al, 2005). Following the establishment of their inhibitory profile, we carried out detailed kinetic studies to determine their mechanism of action.

Compounds	IC50 (μM)
Celecoxib	1.71
18010	10.0
18020	20.9
18030	10.6
18040	>1
18050	40.0
18100	3.0
18110	65.0
18130	30.0
18140	>100

Table 1: IC50 values of SKU-46 series compounds determined using the COX-2 enzyme. All values represent the average of two independent experiments.

Time-dependent inactivation of COX-2 by the 18000 series of compounds: To determine whether the inactivation of COX-2 by the 18000 series of compounds is time-dependent or independent, different concentrations of inhibitor were incubated with the COX-2 enzyme in a reaction buffer. At different time intervals, samples were withdrawn and measured for remaining COX-2 activity as described above. The COX-2 inhibition patterns of Celecoxib, ON018010, ON018030 and ON018100 are shown in **Figure 1**. At the 100μM concentration, all three inhibitors exhibited greater than 50% inhibition of COX-2 in a 10 minute period of time, whereas a relatively slow inhibitory pattern was obtained at the 1μM concentration. For instance, maximal inhibition of COX-2 was achieved within 30 minutes using 15μM of ON018010, ON018030 and ON018100. Approximately 10% of the residual activity was lost after a 50 minute incubation with ON018100 at the 1μM concentration, while ON018100 inhibited approximately 50% of COX-2 activity at the 5μM concentration. These results suggest that the inhibitory activity of all three compounds is time-dependent.

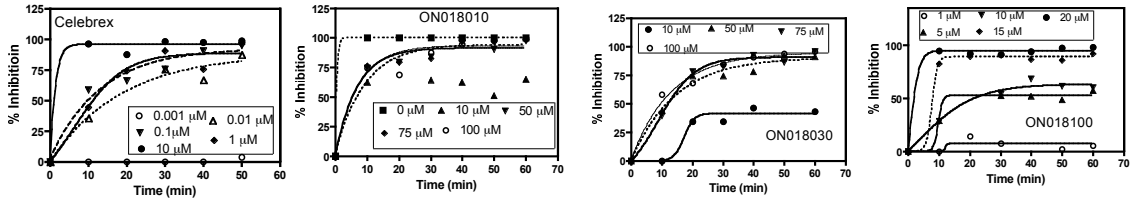


Figure 1: Time dependent inhibition of COX-2 by Celecoxib, ON018010, ON018030 and ON018100, plotted as a non-linear regression curve. Assays were performed as described in the text.

To determine whether the inhibitory of activity of the three compounds is due to covalent binding of the drugs to COX-2, a known concentration of each inhibitor was incubated in the presence and absence of COX-2 for 40 min at room temperature. The COX-2-inhibitor complex was then denatured and the released inhibitor was extracted from the complex by adding one volume of 1:1 (vol/vol) methanol/acetonitrile to the reaction mixture. The solutions were transferred into Micron 30 microconcentrators (Millipore), centrifuged for 5 min in a microcentrifuge and the denatured COX-2 was separated. The

filtrate was dried in a speed Vac (Savant) to remove volatile liquid and solvent. The dried material was dissolved in DMSO and used for the determination of COX-2 inhibitory activity. The inhibitors, recovered from the time dependent inhibition reactions in presence or absence of COX-2 displayed a similar COX-2 inhibition pattern, indicating that the inhibitors were not modified when binding to COX-2 (**Fig. 2**). This suggests that binding between the inhibitors and COX-2 was non-covalent in nature. These results correlate with previous studies conducted for other COX-2 inhibitors such as Dup-697, NS-396 and DFU (Reindeau et al., 1997; Copland etal.,1994).

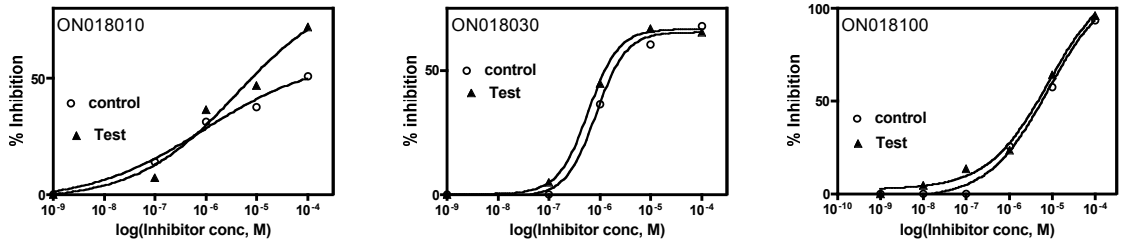


Figure 2: Recovery of inhibitory potency of ON018010, ON018030 and ON018100 after incubation with (○) and without (▲) COX-2 enzyme.

To determine whether the inactivation of COX-2 by the 18000 series of compounds was reversible or irreversible, a known concentration of COX-2 was incubated with various concentrations of inhibitor in a 200 µl of reaction buffer at room temperature. After 40 min, the reaction mixture was loaded into microdialysis chambers (Pierce) with a 10-kDa molecular mass cut-off and dialyzed against 10,000 volumes of inhibitor free reaction buffer for 6 hrs at 4°C. After dialysis, the remaining COX-2 activity in the sample was measured as described above. In the control samples, DMSO, instead of inhibitors, was used (Copeland, 1994b). The activity of the dialyzed COX-2 inhibitor complex was measured simultaneously with the undialyzed COX-2-inhibitior complex.

Our results show that the COX-2 enzyme that was recovered from the dialyzed experiment showed no activity, indicating that the inhibition of COX-2 by ON018010, ON018030 and ON018100 was irreversible (**Fig 3**). Taken together, these studies suggest that the mechanism of COX-2 inhibition by the 18000 series of compounds is time-dependent, non-covalent and irreversible.

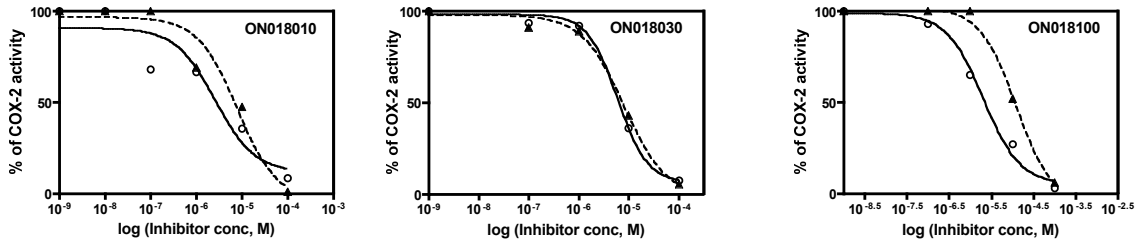


Figure 3: Recovery of COX-2 activity after incubation with different concentrations of ON018010, ON018030 and ON018100. (○) Undialyzed and (▲) dialyzed COX-2.

Effect of the 18000 series compounds on breast tumor cell viability: To test the anti-tumor effects of these compounds, we examined the growth of COX-2 positive and

negative breast tumor cell lines in the presence and absence of the 18000 series compounds. The results of these studies showed that the 18000 series compounds ON018010, ON018030 and ON018100 were active in inducing apoptosis of the COX-2 positive breast cancer cell line, HTB-126. The GI50 values obtained with HTB-126 for Celecoxib was 27uM, while the GI50 for ON018010 was 16uM. The GI50 values for ON018030 and ON018100 were 30 and 22 µM, respectively (**Fig. 4**). When a COX-2-negative breast tumor cell line, BT-20 was used in these assays, Celecoxib had a GI_{50} value of 11 µM while ON018010, 18030 and 180100 exhibited GI50 values of 31µM, 35 µM and 8 µM, respectively (**Fig 5**). Because all of these compounds induce cell death in both COX-2 positive and negative tumors, we conclude that the *in vitro* apototic activity of these compounds could be due to inhibition of COX-2 as well as a second enzyme that is distinct from COX-2.

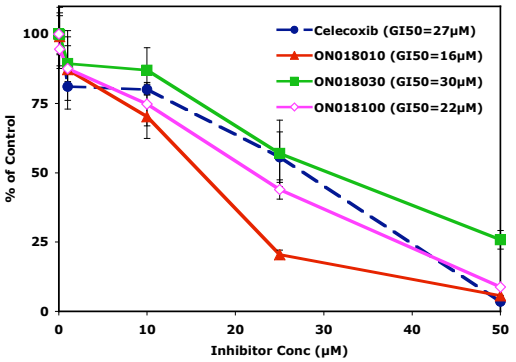


Figure 4: Effect of Celecoxib, ON01810, ON018030 and ON018100 on the proliferation and viability of the COX-2 positive human breast cancer cell line, HTB-126. The cells were cultured with the indicated concentration of each compound for 96 hrs. The number of viable cells was determined by trypan blue exclusion and the average of the three independent experiments was expressed as percentage of vehicle alone (DMSO) treated control samples, which are depicted as 100% growth.

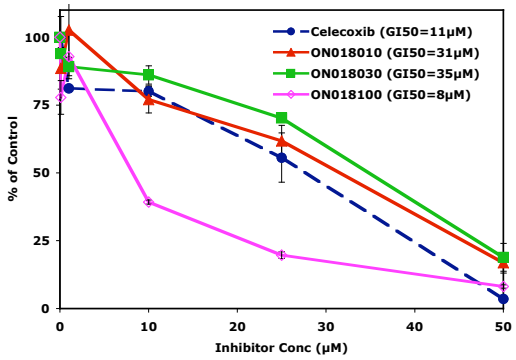


Figure 5 : Effect of Celecoxib, ON01810, ON018030 and ON018100 on the proliferation and viability of the COX-2 negative human breast cancer cell line, BT-20. The cells were cultured with the indicated concentration of each compound for 96 hrs. The number of viable cells was determined by trypan blue exclusion and the average of the three independent experiments was expressed as percentage of vehicle alone (DMSO) treated control samples, which are depicted as 100% growth

Studies with 9000 Series Compounds: The inhibitory effect (reported as the IC_{50} value) of these drugs against the COX-2 (ovine) enzyme was analyzed using a COX inhibitory screening assay kit as described by the manufacturer (Cayman Chemicals, MI) (Gierse et al, 1999). Celecoxib, which has an IC_{50} of 1.71µM was used as a control in all assays. The results of these studies showed that two compounds, 9310A and 9250A were the most potent inhibitors in this series. **Figure 6** shows the inhibitory profiles of ON09250M, ON09250A, ON09250B, ON09310M, ON09310A and ON09310B on COX-2.

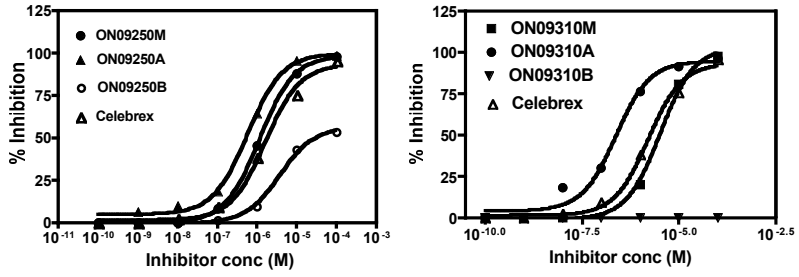


Figure 6: The inhibitory profiles of ON09250M, ON09250A, ON09250B, ON09310M, ON09310A and ON09310B on COX-2. Assays were performed as described in the text.

ON09250A and ON09250B are enantiomers of ON09250M; ON09310A and ON09310B are enantiomers ON09310M. Our results show that the IC50 values for ON09250A were lower than the values obtained for ON09250M and ON9250B, indicating that the inhibitory activity of ON09250M improved after the two optical enantiomers were separated (**Fig 6 & Table 2**).

Table 2: IC50 values for the ON09000 drug series

Compounds	IC50 (μM)
Celebrex	1.86
ON09250M	1.20
ON09250A	0.50
ON09250B	30.20
ON09310M	4.00
ON09310A	0.24
ON09310B	0.00

Similarly, the two optical isomers of ON09310M were separated into enantiomer A (ON09310A) and enantiomer B (ON09310B). Enantiomer A was 6 times more active than Celecoxib at inhibiting COX-2 while enantiomer B displayed no inhibitory activity against COX-2 (**Fig. 6 & Table 2**).

The time dependent inactivation of COX-2 by Celecoxib, ON0920A and ON09310A is illustrated in **Figure 7**. At the 10μM concentration, all three inhibitors exhibited greater than 90% inhibition of COX-2 in a 10 minute period of time, whereas a relatively slow binding pattern was obtained at the

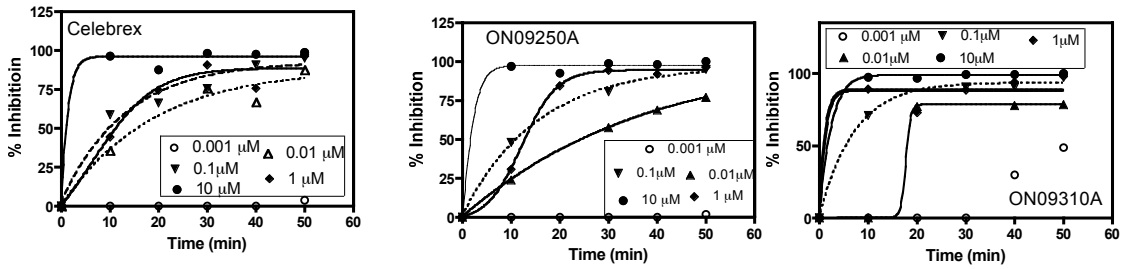


Figure 7: Time dependent inactivation of COX-2 by Celecoxib, ON09250A and ON09310A. Assays were performed as described in the text.

10nM concentration. For instance, maximal inhibition of COX-2 was achieved within 10 minutes with 1μM of ON9310A, whereas at a similar concentration, celecoxib and ON09250A required 30 min. Approximately 10% of the residual activity was lost after a 50 minute incubation with celecoxib or ON09250A at the 1nM concentration while ON09310A inhibited approximately 50% of COX-2 activity under similar conditions. This indicates that compared with celecoxib and ON9250, ON09310A is the most active COX-2 inhibitor at the low concentration of 1nM. Furthermore, COX-2 was completely inhibited after a 10 minute incubation with celecoxib, ON0920A or ON09310A at a concentration greater than 10μM (data not shown).

We also determined the observed rate constant (k_{obs}) for these three inhibitors (**Fig. 8**). The k_{obs} values were calculated using a non-linear least square fit method from the first order decay curve for different concentrations of inhibitor. The k_{obs} values at the range of inhibitor concentrations studied for ON9310A were significantly higher than that of Celecoxib and ON9250A. These kinetic analyses also indicate that ON09310A is the most effective COX-2 inhibitor compared to Celecoxib and ON9250A. The mechanism

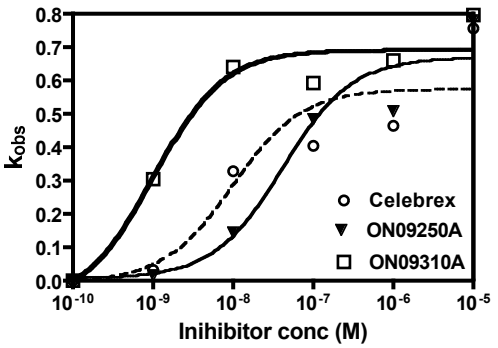


Figure 8: Concentration dependence of the observed rate constant (k_{obs}) of COX-2 inactivation for celecoxib, ON09250A and ON09310A. The curve represents non-linear least square fits. Assays were performed as described in the text.

of inhibition of COX-2 by Celecoxib, ON09250A and ON09310A can be described as "time dependent," involving a tight binding between the inhibitor and enzyme. This time dependent factor is considered as one of the basic characteristics for the selectivity of novel COX-2 inhibitors (Gierse et al., 1996).

To investigate the inhibition pattern of celecoxib, ON9250A and ON9310A, each compound and substrate were equilibrated in the reaction mixture before adding COX-2. The effect of various substrate concentrations on the inhibition of COX-2 by the three inhibitors, Celecoxib, ON09250A and ON09310A is shown in **Figures 9 - 11**. The inhibition data were fit into an equation for competitive inhibition (v= VmaxS/Kmapp(1 + I/Ki)+ S) (Tian et al., 2002) and non-linear regression analysis was performed.

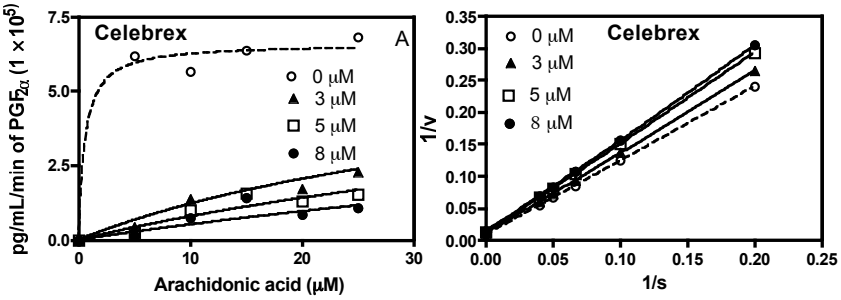


Figure 9: Effect of substrate concentrations on the inactivation of COX-2 by Celebrex (A). Reciprocal plot of COX-2 inactivation by Celebrex (B). Assays were performed as described in the text.

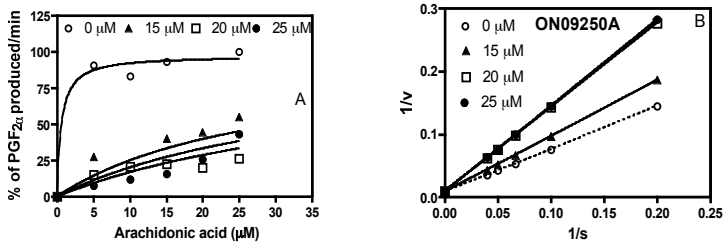


Figure 10: Effect of substrate concentrations on the inactivation of COX-2 by ON09250A. (A) Regression analysis. (B) Reciprocal plot of COX-2 inactivation by ON09250A. Assays were performed as described in the text.

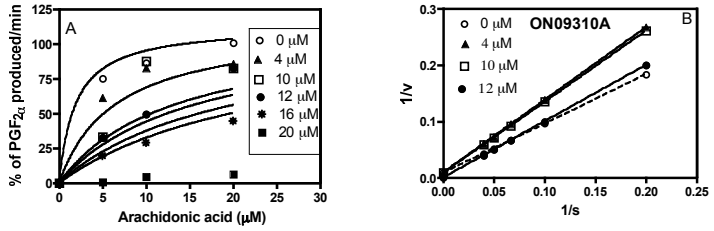


Figure 11: Effect of substrate concentration on the inactivation of COX-2 by ON09310A. (A). Regression analysis. (B) Reciprocal plot of COX-2 inactivation by ON09310A. Assays were performed as described in the text.

The results of these studies show that with increasing substrate concentration, the loss of COX-2 activity was decreased. This indicates that the inhibitors compete with the substrate for binding to COX-2. In addition, the rate of $PGF_{2\alpha}$ synthesis decreased with increasing concentrations of inhibitors resulting in increased Km_{app} and unchanged V_{max} values. This result further demonstrates the competitive nature of these inhibitors. Our results also showed that pre-

incubation of COX-2 with any one of these inhibitors for 60 minutes reduced the ability of the substrate to compete with the inhibitor for the active site. These results were further analyzed using a double reciprocal plot of 1/v versus 1/s derived using the Michaelis-Menten equation. We observed that the $1/Km_{app}$ values increased with increasing concentrations of inhibitors. However, there were no changes in the 1/Vmax (y-intercept). These results allow us to conclude that the inhibition of COX-2 by celecoxib, ON9250A and ON9310A was competitive. Furthermore, celecoxib, ON09250A and ON09310A displayed characteristics of irreversible competitive inhibitors.

The inhibitors, when recovered from the time dependent inhibition reactions in presence or absence of COX-2, displayed similar a COX-2 inhibition pattern, indicating that the inhibitors were not modified when binding to COX-2 (**Fig. 12**). Therefore, these inhibitors have retained their inhibitory potency against COX-2, which suggests that binding between them and COX-2 was non-covalent in nature. Our results for the

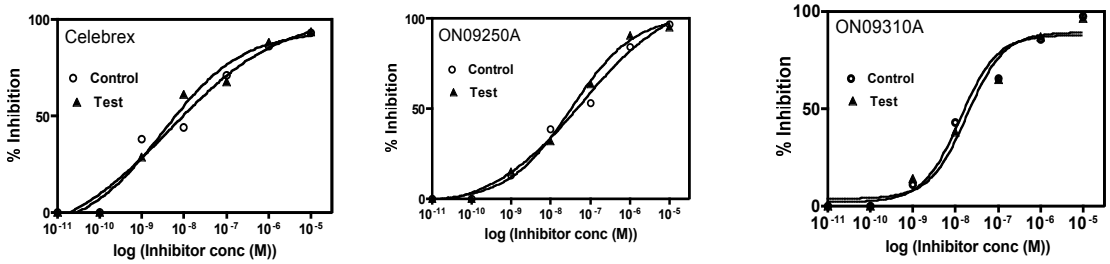


Figure 12: Recovery of Inhibitory potency of Celecoxib, ON09250A and ON09310A. Inhibitors were incubated with (Test) and without (Control) COX-2 enzyme and extracted from the complex as described in the text and assayed for COX-2 inhibitory activity.

recovery of inhibitory potency also correlate with previous studies conducted for selective COX-2 inhibitors such as Dup-697, NS-396 and DFU (Reindeau et al., 1997; Coplandetal.,1994). The COX-2 enzymes that were recovered from the dialyzed experiment showed no activity, indicating that the inhibition of COX-2 by celecoxib, ON9250A and ON09310A was irreversible (**Fig. 13**). Walker et al., (2001) reported that this type of irreversible inactivation between the inhibitors and COX-2 involved a conformational change in the active site of COX-2. Our kinetic data obtained for

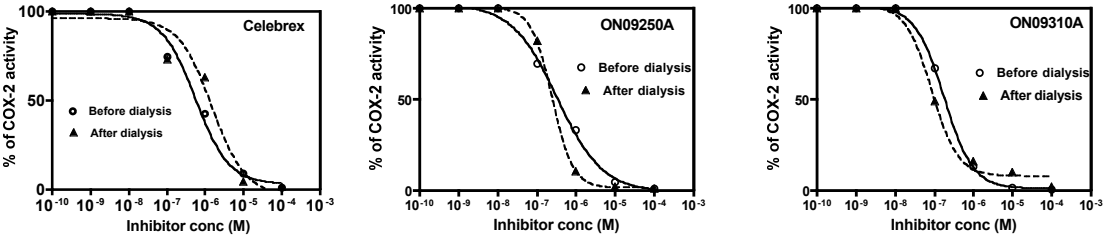


Figure 13: Recovery of COX-2 activity after 40 min incubation with Celecoxib, ON09250A and ON09310A.

ON09250A and ON09310A are similar to celecoxib and other selective COX-2 inhibitors, and suggest that upon binding to COX-2 active site, these inhibitors induce irreversible conformational change of the enzyme.

Molecular Modeling Analysis: In order to establish the molecular basis for the superior activity of our COX-2 inhibitors, and to determine the active site interactions of the isomers, we performed molecular modeling analysis as detailed below. These studies indicate the enhanced activity of one isomer over the other is likely due to improved hydrogen binding of the inhibitor at the active site of the enzyme. We are currently employing this methodology to aid in the design of new compounds in this class with superior COX-2 inhibitory activity.

All molecular modeling was performed on a Silicon Graphics Personal IRIS 4D/25 workstation. X-ray crystal structures for COX-1 (1PGF) and COX-2 (4COX) described by Loll and coworkers (1995) and Kurumbail and coworkers (1996), respectively were obtained from the Protein Data Bank (Berman et al, 2000). All calculations were performed using the DREIDING II all atom force field and Biograf® software (BIOSYM/Molecular Simulations, San Diego, CA). Modified crystal structures for COX-1 and COX-2 were obtained by the addition of all heterogeneous hydrogen atoms in optimized positions according to the Biograf® software protocols.

Models of the anti-inflammatory drug analogs examined, i.e., celecoxib, ON09250 and its enantiomers, were constructed using the organic builder contained within the main Biograf® program and were individually docked into the active site of COX-2 by overlaying the structure of each analog with the structure of indomethacin, derived from the original crystal structure. Modeling calculations were performed only allowing for the movement of each analog within the active site, while the atomic coordinates of COX-2 were held constant. The calculations involved energy minimization to convergence, limited molecular dynamics calculations (5 psec.), followed again by energy minimization to convergence. By overlaying the crystal structure of COX-1 with COX-2, the models of the analog:cyclooxygenase complexes could be evaluated for binding efficiency to COX-2 by evaluating hydrogen bonding interactions and for a lack of binding efficiency to COX-1 as a result of the steric hindrance in COX-1 due to the presence of the longer side chain of Ile 523 compared to V523 in COX-2.

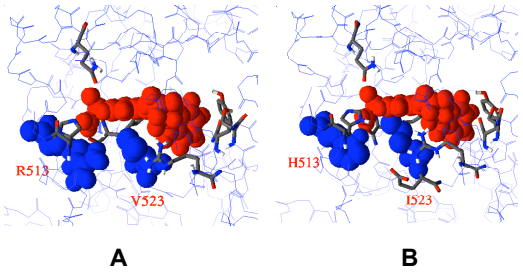


Fig. 14. Steric selectivity of COX-2 analogs. The protein main chain for COX-2 (A) and COX-1 (B) is shown in light blue. Space filling models of ON09250 and residues involved in isotype selectivity are shown in red and blue respectively.

All cyclooxygenase inhibitors examined were sterically selective for COX-2 over COX-1. As an example, the presence of Arg 513 and Val 523 in COX-2 do not inhibit the binding of analog ON09250 (**Figure 14A**), while the presence of His 513 and the longer side chain of Ile 523 in COX-1 resulted in increased van der Waals interactions accounting for the steric inhibition of the binding of ON09250 to COX-1 (**Figure 14 B**). Examination of the molecular models for each analog docked into COX-2 established a rubric for assessing the binding efficiency of COX-2 specific analogs based on the number and type of the possible hydrogen bonding interactions suggested by the models. The data shown in **Figure 15**

was constructed by analyzing active site snapshots of molecular models of analog:COX-2 complexes. Note the absence of hydrogen bonding interactions to His 90 and Q192 when celecoxib was docked with COX-2 (**Figure 15**) compared to the complex containing ON09250 (enantiomer A) docked with COX-2 (**Figure 15**).

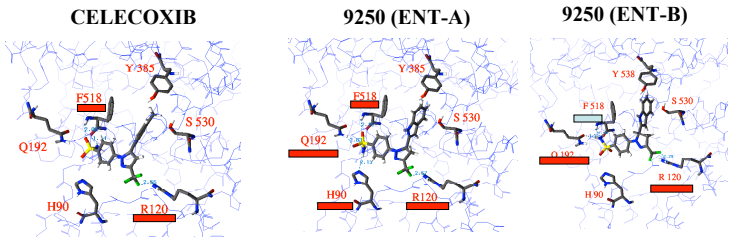


Fig. 15. Hydrogen Bonding Interaction of Celecoxin and ON09250 at the COX-2 Active Sit. Active Site Snapshots of Molecular Models of Analog:COX-2 complexes. The main chain atoms of the COX-2 protein are shown in blue. Residues previously demonstrated to be involved in ligand binding are identified. A: celecoxib, B: ON09250 (enantiomer-A), C: ON09250 (enantiomer-B). Hydrogen bonds are represented as dotted lines.

Analog	Q192	H90	R120	F518(αNH$_2$)
ON09250(ent1)	2.83A(SO$_2$NH$_2$)[1]	2.13A(SO$_2$NH$_2$)	2.56A(CF$_3$)	2.15A(SO$_2$NH$_2$)
ON09250(ent2)	1.91A(SO$_2$NH$_2$)	***	2.29A(CF$_3$)	2.58A(SO$_2$NH$_2$)
Celecoxib	***	***	2.55A(CF$_3$)	1.94A(SO$_2$NH$_2$)

Because a chiral center is present in ON09250 at the point of attachment of the pyrrole group (see **Figure 16**), this analog exists in two different enantiomeric forms with the pyrrole ring pointing out (enantiomer-A) or in (enantiomer-B) as shown in **Figure 16.** Initial docking of ON09250 (enantiomer-B) with the COX-2 protein resulted in major van der Waals contacts between the ligand and residues Glu 524, Arg 120, and Val 523 due to the opposite orientation of the pyrrole group on ON09250 (enantiomer-A). In order to successfully dock this analog into the active site of COX-2, the intact enantiomer-B was rotated to more closely align the pyrrole ring of this enantiomer with that of enantiomer-A as shown in **Figure 16**. The molecular model of the resulting enantiomer-B:COX-2 complex was subjected to energy calculations and its active site snapshot is shown in **Figure 15**. Comparison of **Figure 15** with **Figure 15** suggests that the binding efficiency of the ON09250 enantiomer-B should be less than that of enantiomer-A for two reasons. First, a hydrogen bond to His 90 is not observed when enantiomer-B was docked into the COX-2 active site. In addition, in the presence of COX-2, the hydrogen bonds demonstrated to be present between enantiomer-B and Gln 192 or Phe 518 involve the less electronegative sulfamide NH_2 group as compared to the more electronegative sulfamide SO_2 group in the presence of enantiomer-A.

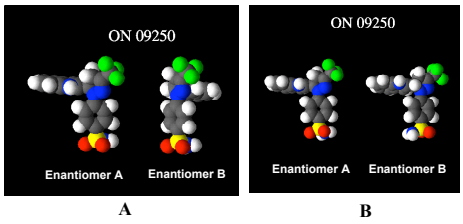


Figure 16. Molecular models of the enantiomeric forms of analog ON09250. Molecular models are shown for the enantiomers of ON09250 before (A) and after (B) docking into the COX-2 protein.

Effect of the 9000 series compounds on breast tumor cell viability: To test the anti-tumor effects of these compounds, we examined the growth of COX-2 positive and negative cell lines in the presence and absence of the 9300 series compounds. The results

of these studies showed that ON09250A and ON09310A showed 1.8 and 4.5-fold more cytotoxic effect on HTB-126 cells than celecoixb (**Fig. 17**). Similarly, cytotoxicity studies using the COX-2 negative BT-20 cell line showed that ON09310A was 2-fold more potent than celecoxib in this system. Because all of these compounds induce cell death in both COX-2 positive and negative tumors, we conclude that the *in vitro* apototic activity of these compounds could be due to inhibition of an enzyme that is distinctive from COX-2.

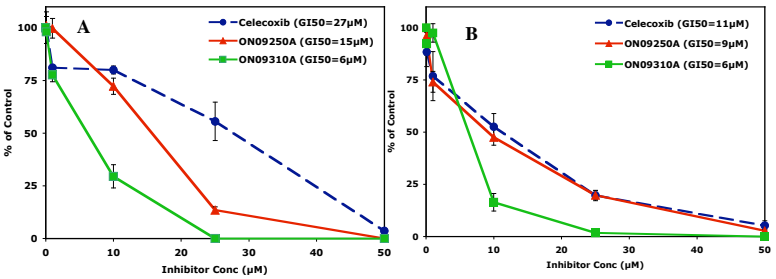


Figure 17.(A) Effect of celecoxib, ON019250A and 9310A on the cell proliferation and viability of the COX-2 positive human breast cancer cell line, HTB-126. The cells were cultured with the indicated concentration of each compound for 96 hrs. The number of viable cells was determined by trypan blue exclusion and the average of the three independent experiments was expressed as percentage of vehicle alone (DMSO) treated control samples, which are represented as 100% growth. (B) Effect of Celecoxib, ON09250A and 9310A on the cell proliferation and viability of COX-2 negative human breast cancer cell line, BT-20. The cells were cultured with the indicated concentration of each compound for 96 hrs. The number of viable cells was determined by trypan blue exclusion and the average of the three independent experiments was expressed as percentage of vehicle alone (DMSO) treated control samples, which are represented as 100% growth

Effects of compounds ON09250A and ON09310A on cell cycle progression: As stated above, ON09250A and ON09310A are extremely potent COX-2 inhibitors. Their IC_{50} values of 0.85μM and 0.29μM, respectively, are 2-5.9 fold more potent than celecoxib, which has an IC_{50} value of 1.71μM. To determine the effects of these compounds on cell cycle progression, COX-2 positive and negative tumor cell lines were synchronized at late G_1/S phase with aphidicolin (1μg/ml). After 24 hours, the media was replaced with fresh medium containing DMSO (control) or ON09250A or ON09310A. Cells were collected at 0, 24, 48 and 72 hour time points and the cell cycle distribution determined by flow cytometric analysis. As expected, the control-treated cells re-entered the cell cycle after the removal of aphidicolin. Similar results were obtained in cells that were treated with ON09250A, although a greater percentage of cells remained in G_1 throughout the experiment. However, at the 24hour time point, cells that were treated with ON09310A remained arrested in the G1 phase. In addition, a noticeable percentage of the cells were beginning to enter the apoptotic pathway. At the 48-hour time point, the number of apoptotic cells had doubled, and by 72 hours, nearly all of the cells were apoptotic. These studies show that 9310A induces an irreversible G_1 arrest that ultimately leads to apoptosis.

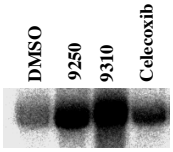


Fig. 18. Upregulation of DR-5 mRNA by COX-2 inhibitors. COX-2 positive tumor cells were treated with 40 μM concentration of each of the indicated drug or DMSO (Vehicle Control) for 8 Hrs and the total RNA examined for DR-5 transcripts by Northern blot analysis

Upregulation of DR-5 transcription by ON09310: We next investigated the mechanism by which ON09310 upregulates DR5 expression and as shown in **figure 18**, ON09310 increases DR5 m RNA levels within 8 Hrs following the treatment of cells with the drug. Celecoxib induced moderate levels of DR-5 mRNA while ON09250 and ON09310 were potent inducers of DR-5 transcripts. These results indicate that ON09310 up-regulates DR5 levels by increasing the DR5 gene transcription.

Synergism between TRAIL and ON09310 to induce apoptosis: Because ON09310 up-regulates DR5, we next investigated whether TRAIL, the ligand for DR5, would sensitize cells to ON09310. As shown in **figure 19**, high concentrations of TRAIL were required to induce apoptosis of HCT15 cells, and in combination with ON09310, TRAIL promotes apoptosis in these human tumor cells within 8 hrs at lower concentrations. We also investigated the effect of these agents on the activation of caspases 3, 8 and 9 under similar experimental conditions. Our results show that the combination of TRAIL and ON09310 strongly induces the activation of these caspases this tumor cell model system (data not shown). Together, these results highlight the functional significance of ON09310-mediated up-regulation of DR5.

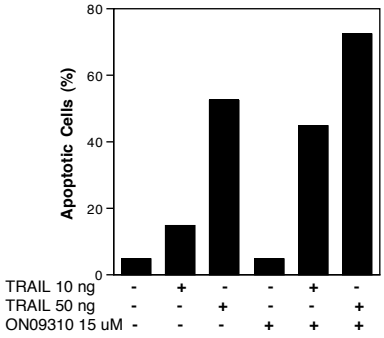


Fig. 19. ON09310 potentiates TRAIL-induced apoptosis of COX-2-positive tumor cells. Time of Treatment is 8 hours

KEY RESEARCH ACCOMPLISHMENTS

We have synthesized two new classes of COX-2 inhibitors that possess tumor growth inhibitory activity. These new compounds inhibit the growth of both COX-2 positive and negative cells, suggesting that they may target other protein(s) that play an important role in tumor cell proliferation. We have also determined that our most potent COX-2 inhibitor, which is almost 6-fold more active than Celecoxib, induced G_1 arrest of tumor cells and ultimately activates their apoptotic pathway.

REPORTABLE OUTCOMES

Boominathan, R., Ramana Reddy, M.V., Cosenza, S.C., Mallireddigari, M., Sheikh, S. and Premkumar Reddy, E. Novel COX-2 inhibitors-II with enhanced anti-tumor activity. Paper presented at Era of Hope-Department of Defense Breast Cancer Research Program Meeting, 2005, Philadelphia, USA.

Ramana Reddy, M.V., Muralidhar Reddy, M., Venkant, R.P., Padmavathi, V. Boominathan, R. Stanley, C.B. and Premkumar Reddy, E.P. (2005) Design, synthesis, and biological evaluation of (E) and (Z)-styryl-(2)-acetoxyphenyl sulfides and sulfones as cyclooxygenase-2 inhibitors. *Bioorganic and Medicinal Chemistry* 13, 1715-1723.

Boominathan R., Reddy M.V.R., Cosenza, S.C., Sheikh, M.S. and Reddy, E. P. 2004. Novel COX-2 Inhibitors with Enhanced Anti-tumor Activity. 20th Annual Meeting on Oncogenes, Frederick MD, June 2004.

Pallela, V.R., Boominathan, R., Venkatapuram, P, Reddy, E. P. and Reddy, M.V.R. 2004. Synthesis of Styryl Acetophenylsulfides: Novel Cyclooxygenase Inhibitors. 227th ACS National Meeting.

Boominathan R., Reddy M.V.R., Cosenza, S.C., R. Dhanasekaran and Reddy, E. P. 2004. Kinetic analysis of COX-2 inhibition by (Z)-styryl-(2)-acetoxyphenyl sulfides and sulfones. Biochemistry (submitted).

Boominathan, R., Pallela, V.R., Venkatapuram, P., Reddy, M.V.R., Dhanasekaran, R. and Reddy, E.P. Kinetic analysis of COX-2 inhibition by Styryl Acetophenylsulfides. J. Biolo. Chemistry (Submitted).

CONCLUSIONS

The involvement of COX-2 in breast tumor growth has necessitated the development of specific COX-2 NSAIDs. In terms of breast cancer therapy, it is necessary to develop new therapeutic agents that possess both growth inhibitory and pro-apoptotic properties that are more efficacious than the present group of drugs (which were originally developed to treat inflammation). Our results to date show that we have developed novel agents that inhibit COX-2 and have growth inhibitory and pro-apoptotic activities against breast tumor cells. These studies suggest that these compounds may play an important role as anti-cancer and chemopreventive agents.

These studies help establish the basis for the specificity and superior activity of ON09250A and 9310A as COX2 inhibitors. The main conclusions from these studies are:

1. ON09250 and ON09310 fit the active site of COX2 enzyme with multiple hydrogen bonding contact with key residues within the active site.

2. Isoform A appears to interact with the critical residues better than Isoform B both these compounds, suggesting that the isolated product would be preferable for further studies.

3. A Comparison of interactions of these inhibitors in the active sites of COX-1 and COX-2 helps rationalize the basis for the high selectivity of these compounds.

REFERENCES

Alshafie, G.A., Abou-Issa, H.M., Siebert, K. and Harris, R.E. (2000). Chemotherapeutic Evaluation of Celecoxib, a Cyclooxygenase-2 Inhibitor, in a Rat Mammary Tumor Model. Oncol. Rep. 7: 1377-1381.

Badawi, A.F., El-Sohemy, A., Stephen, L.L., Ghoshal, A.K. and Archer, M.C. (1998). The effect of Dietary n-3 and n-6 Polyunsaturated Fatty Acids on the Expression of Cyclooxygenase 1 and 2 and Levels of p21ras in Rat Mammary Glands. Carcinogenesis 19: 905-910.

Copeland, RA, Williams, JM., Giannaras, J., Nurnberg, S., Covington, M., Pinto, D., Pick, S. and Trzaskos, JM. (1994) Mechanism of selective inhibition of the inducible isoform of prostaglandin G/H synthase. Proc. Natl. Acad. Sci. USA., 91, 11202-11206.

Gierse, J.K., Koboldt, C.M., Walker, M.C., Seibert, K. and P.C. Isakson (1999) Kinetic basis for selective inhibition of cyclooxygenases. The Biochemical Journal, 339, 607-614.

Gierse, JK., McDonald, JJ., Hauser, SD., Rangwala, SH., Koboldt, CM. and Seibert, K. (1996) A single amino acid difference between cyclooxygenase-1 (COX-1) and –2 (COX-2) reverse the selectivity of COX-2 specific inhibitors. The J Biol. Chem. 271, 15810-15814.

Krumbali, R.G., Stevens, A.M., Gierse, J.K., McDonald, J.J., Stegeman, R.A., Pak, J. Y., Gidehaus, D., Miyasshiro, J.M., Penning, T.D., Siebert, K., Iskason, P.C. and Stallings, W.C. (1996). Structural basis for selective Inhibition of COX-2 by anti-inflammatory agents. Nature 384: 344-348.

Liu, X.H. and Rose, D.P. (1996). Differential Expression and Regulation of Cyclooxygenase-1 and 2 in Two Human Breast Cancer Cell Lines. Cancer Res. 56: 5125-5127.

Loll PJ, Picot D and Garavito RM (1995) The structural basis of aspirin activity inferred from the crystal structure of inactivated prostaglandin H2 synthase. *Nat Struct Biol* **2:** 637-643.

Prescott, S.M. and Fitzpatrick, F.A. (2000). Cyclooxygenase-2 and Carcinogenesis. Biochim. Biophys. Acta 1470: M69-78.

Reddy, R. M.V., Reddy, M. M., Venkant, R.P., Padmavathi, V. Boominathan, R. Bell, S.C. and Reddy, E.P. (2005) Design, synthesis, and biological evaluation of (E) and (Z)-styryl-(2)-acetoxyphenyl sulfides and sulfones as cyclooxygenase-2 inhibitors. *Bioorganic and Medicinal Chemistry* 13, 1715-1723.

Riendeau, D., Percival, M.D., Boyce, S., Brideau, C., Charleson, S., Cromlish, W., Ethier, D., Evans, J., Falgueyret, JP., Ford-Hutchinson, AW., Gordon, R., Greig, G., Gresser, M., Guay, J., Kargman, S., Leger, S., Mancini, JA., O'Neill, G., Ouellet, M., Rodger, IW., Therien, M., Wang, Z., Webb, JK., Wong, E., Xu, L., Young, RN., Zamboni, R., Prasit, P. and Chan, CC. (1997) Biochemical and Pharmacological profile of a tetrasubstituted furanone as a highly selective COX-2 inhibitor. Br J Pharmacol., 121, 105-117.

Soslow, R.A., Dannenberg, A.J., Rush, D., Woerner, B.M., Khan, K.N., Masferrer, J. and Koki, A.T. (2000). COX-2 is Expressed in Human Pulmonary, Colonic and Mammary Tumors. Cancer 89: 2637-2645.

Taketo, M.M. (1998). Cyclooxygenase Inhibitors in Tumorigenesis. Part I. J. Natl. Cancer Inst. 90: 1529-1536.

Taketo, M.M. (1998). Cyclooxygenase Inhibitors in Tumorigenesis. Part II. J. Natl. Cancer Inst. 90: 1609-1620.

Tian, G., Sobotka-Briner, CD., Zysk, J., Liu, X., Birr, C., Sylvester, MA., Edwards, PD., Scott, CD., Greenberg, BD. (2002) Linear non-competitive inhibition of solubilized human γ-secretase by pepstatin A methylester, L685458, sulfonamides, and benzodiazepines. J. Biol. Chem., 277, 31499-31505.

Vane, J.R. and Botting, R.M. (1998). Overwiew: The Mechanism of Action of Anti-inflammatory Drugs. In "Clinical Significance and Potential of Selective COX-2 Inhibitors." Vane, J. and Botting, R., eds. William Harvey Press, UK, 1-17.

Walker, MC., Kurumbail, RG., Kiefer, JR., Moreland, KT., Koboldt, CM., Isakson, PC., Seibert, K. and Gierse, JK (2001) A three-step kinetic mechanism for selective inhibition of cyclooxygenase-2 by diarylhetrocyclic inhibitors. Biochem. J., 357, 709-718.

Williams, C.S. Mann, M. and DuBois, R.N. (1999). The Role of Cyclooxygenases in Inflammation, Cancer and Development. Oncogene 18: 7908-16.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry 13 (2005) 1715–1723
Bioorganic & Medicinal Chemistry

Design, synthesis, and biological evaluation of (*E*)- and (*Z*)-styryl-2-acetoxyphenyl sulfides and sulfones as cyclooxygenase-2 inhibitors

M. V. Ramana Reddy, Muralidhar Reddy Mallireddigari, Venkat R. Pallela, Padmavathi Venkatapuram, Rengasamy Boominathan, Stanley C. Bell and E. Premkumar Reddy*

Fels Institute for Cancer Research, Temple University School of Medicine, 3307 North Broad Street, Philadelphia, PA 19140-5101, USA

Received 29 September 2004; revised 2 December 2004; accepted 2 December 2004
Available online 25 December 2004

Abstract—A new series of styryl acetoxyphenyl sulfides and sulfones possessing (*E*)- and (*Z*)-configurations were designed and prepared by stereospecific syntheses. All these compounds were evaluated for their ability to inhibit COX-2 enzyme in vitro. Structure–activity relationship studies on these compounds revealed that only sulfides with (*Z*)-configuration have potential COX-2 inhibitory activity. This inactivation of the enzyme is believed to be due to the selective covalent modification of COX-2 by the inhibitors.

1. Introduction

Cyclooxygenases (COXs) are key enzymes in the synthesis of prostaglandin H_2, which is a precursor for the biosynthesis of prostaglandins, thromboxanes, and prostacyclins.[1] COX enzymes exist in two isoforms, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2).[2] COX-1 enzyme is constitutively expressed and is critical for protection of gastric mucosa, platelet aggregation and renal blood flow whereas COX-2 enzyme is inducible and expressed during inflammation, pain, and oncogenesis.[3] Since COX-2 is involved in inflammation and pain, molecules that inhibit it's enzymatic activity would be of therapeutic value. Many nonsteroidal anti-inflammatory drugs (NSAIDs) were found to interact with these enzymes and inhibit their enzymatic activity.[4] These molecules include aspirin and indomethacin which are nonselective and inhibit both COX-1 and COX-2 enzymes. Aspirin inhibits COX-1 more strongly than COX-2[4] and inhibition of COX-1 by aspirin reduces the production of PGE_2 and PGI_2, which has an adverse ulcerogenic effect.[5]

Recently several new inhibitors were developed, which selectively inhibit COX-2 enzyme without interfering with COX-1 enzymatic activity. These molecules include celecoxib,[6] rofecoxib,[7] and valdecoxib,[8] which inhibit COX-2 enzyme without the side effects observed with traditional NSAIDs. These selective inhibitors take advantage of the larger enzymatic pocket in COX-2 active site where valine at 523 has a smaller side chain that accommodates the sulfur containing side chains of the inhibitors, where the isoleucine at 523 in COX-1 has a bigger side chain preventing the docking of the inhibitor at the active site.[9] This preferential binding of selective inhibitors to COX-2 enzyme over COX-1 enzyme prevents the side effects as seen in nonselective inhibitors.[10]

Aspirin is a unique NSAID that interacts with both cyclooxygenases but inhibits COX-1 10- to 100-fold more effectively than the COX-2 enzyme.[11] Aspirin's inhibitory activity is due to its ability to acetylate serine residues positioned at Ser^{530} in COX-1 and Ser^{516} in COX-2.[12,13] Acetylation of these residues prevents the positioning of arachidonic acid to its binding site thereby blocking the substrate to the active site of oxygenation. Because of its dual inhibitory activity, some of

Keywords: Styryl sulfides; Sulfones; COX-2; Structure–activity relationship.
* Corresponding author. Tel.: +1 215 707 4307; fax: +1 215 707 1454; e-mail: reddy@temple.edu

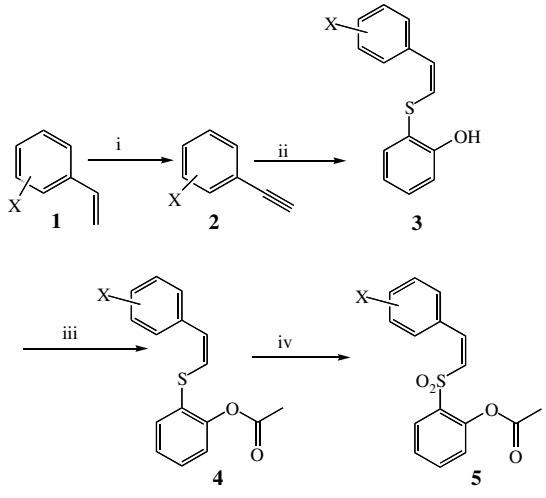
0968-0896/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2004.12.005

aspirin's therapeutic advantages can be attributed to its ability to reduce inflammation by acetylating COX-2 and prevent platelet aggregation and anti-thrombosis by acetylating COX-1.[14] Recent studies[15] on selective COX-2 inhibitors revealed that patients with heart disease are more prone to myocardial infarction and this may be due to TxA_2/PGI_2 imbalance created by selective COX-2 inhibitors.[16] These observations can be exploited in designing a novel aspirin like molecule, which can have all the benefits of COX-2 irreversible inhibition and retaining anti-thrombotic activity by selective acetylation of COX-1 enzyme. Recently, Kalgutkar and co-workers[17,18] have synthesized novel aspirin like analogs with variations at acyl group, alkyl group, aryl substitution pattern and heteroatom substitution and discovered a lead molecule that selectively acetylated, and irreversibly inactivated, COX-2.

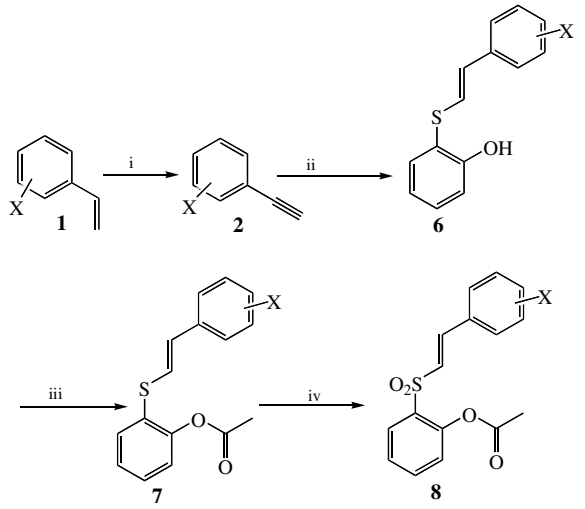
In this paper, we wish to report the synthesis of novel (*E*)- and (*Z*)-styryl-2-acetoxyphenyl sulfides, sulfones, and their selective inhibition of COX-2 enzyme. We believe that these compounds may be inhibiting COX-2 enzyme by modifying the serine residue located in the catalytic domain of the enzyme. The significance of this paper lies in the design and synthesis of new COX-2 inhibitors that may possess both anti-thrombotic activity of aspirin and anti-inflammatory activity of celecoxib and rofecoxib.

2. Chemistry

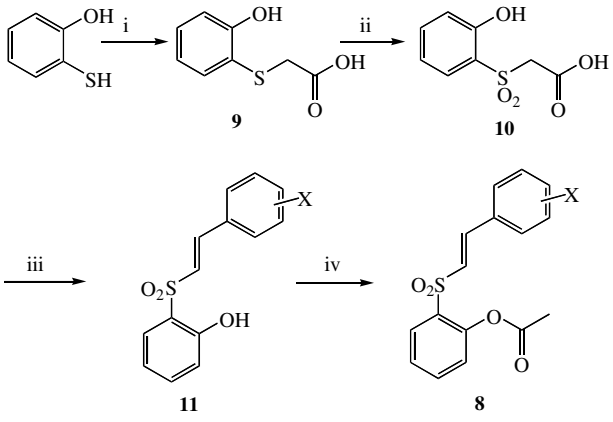
The title compounds were synthesized using the reaction sequence illustrated in Schemes 1–3. Accordingly, bromination and dehydrobromination of styrenes (**1**) in alcoholic potassium hydroxide solution yielded phenylacetylenes (**2**).[19] Nucleophilic addition of the thiol to phenylacetylenes in the presence of a base yielded a (*Z*)-styryl hydroxyphenyl sulfides (**3**) by *trans* addition.[20] Acetylation of (*Z*)-styryl hydroxyphenyl sulfides with acetic anhydride and pyridine gave (*Z*)-styryl acetoxyphenyl sulfides (**4**). Oxidation of these sulfides with hydrogen peroxide[21] in acetic acid resulted in the formation of (*Z*)-styryl acetoxyphenyl sulfones (**5**) (Scheme 1).



Scheme 1. Reagents and conditions: (i) Br_2, CCl_4, 4 °C, KOH, EtOH, reflux 12 h; (ii) 2-hydroxythiophenol, NaOH, MeOH, reflux 24 h; (iii) Ac_2O, pyridine/CH_2Cl_2, 6 h; (iv) 30% H_2O_2, AcOH, 16 h.



Scheme 2. Reagents and conditions: (i) Br_2, CCl_4, 4 °C, KOH, EtOH, reflux 12 h; (ii) 2-hydroxythiophenol, AcOH, $(Ac_2)_3Mn{\cdot}2H_2O$; (iii) Ac_2O, pyridine/CH_2Cl_2, 6 h; (iv) 30% H_2O_2, AcOH, 16 h.



Scheme 3. Reagents and conditions: (i) mercaptoacetic acid, NaOH, MeOH, reflux 5 h; (ii) 30%H_2O_2, AcOH, 16 h; (iii) aromatic aldehyde, benzoic acid, piperidine, toluene, reflux 4 h; (iv) Ac_2O, pyridine/CH_2Cl_2, 6 h.

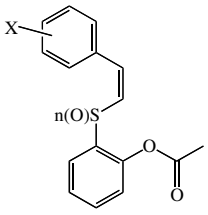
The corresponding (*E*)-isomers of styryl hydroxyphenyl sulfides (**6**) were synthesized by free radical addition of 2-hydroxy thiophenol to phenylacetylene.[22] Acetylation of the resulting sulfide (**7**) followed by the oxidation yielded (*E*)-styryl acetoxyphenyl sulfones (**8**) (Scheme 2). Alternately, (*E*)-styryl acetoxyphenyl sulfones were also synthesized by Knoevenagel type condensation[23] of 2-hydroxyphenylsulfonyl acetic acid (**10**) with various aromatic aldehydes followed by acetylation of the styryl sulfone (**11**) (Scheme 3). The (*E*)- and (*Z*)-configurations to these isomers were assigned by NMR. All (*Z*)-isomers have shown coupling constants between 10 and 11 Hz for their vinylic protons whereas the coupling constants for the vinylic protons in the (*E*)-isomers were found between 15 and 16 Hz.[24]

3. Results and discussion

We have synthesized a series of (*E*)- and (*Z*)-isomers of styryl acetoxyphenylsulfides (**4**, **7**) and sulfones (**5**, **8**) and evaluated their ability to inhibit COX-2 and COX-1 enzymes in vitro by using recombinant COX-1 and COX-2 enzymes. IC_{50} values for inhibition of ovine COX-1 and COX-2 by these compounds were determined by Enzyme Immuno Assay (Tables 1 and 2).[25]

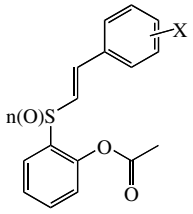
Inhibition of COX-2 enzyme by aspirin is due to its initial binding to an arginine residue (Arg^{499}) by its O-carboxylate group followed by the transfer of an acetyl moiety to the weakly nucleophilic hydroxyl group of the serine residue (Ser^{530} in COX-1 and Ser^{516} in COX-2).[26] Based on this, we have decided to synthesize a molecule capable of transferring an acetyl group to the serine residue to prevent the interaction of arachidonic acid with the COX-2 enzyme. Unlike aspirin, which inhibits COX-1 preferentially over COX-2, most of the (*Z*)-styryl acetoxyphenyl sulfides (**4**) inhibited COX-2 enzyme at lower concentrations as compared to COX-1 showing their specificity toward COX-2 enzyme (Table 1). It has been shown that the fatty acid binding site in COX-2 enzyme is larger than COX-1[27] and therefore designing a molecule that fits in COX-2 pocket and not in the COX-1 would result in specific inhibition of the COX-2 enzyme. Selectivity of (*Z*)-styryl acetoxyphenyl sulfides (**4**) for the COX-2 enzyme inhibition may be due to the presence of the bulkier (*Z*)-styryl thio group *ortho* to the acetate, which may fit well in the COX-2 enzyme pocket while not fitting into the COX-1 active site.

Table 1. In vitro COX-1 and COX-2 inhibition by (*Z*)-styryl acetoxyphenylsulfides and sulfones



Compound	*n*	X	IC_{50} (μM)	
			COX-2	COX-1
Celecoxib	—	—	1.7	>100
Aspirin	—	—	93	15.5
4a	0	H	10.8	>100
4b	0	4-F	18.9	>100
4c	0	2-F	10.6	>100
4d	0	4-Cl	27.9	>100
4e	0	4-Br	39.8	>100
4f	0	4-CH_3	27.8	>100
4g	0	4-CH_2CH_3	46.4	>100
4h	0	4-$(CH_2)_4CH_3$	73.7	>100
4i	0	3-$OCOCH_3$	2.8	>100
4j	0	4-OCH_3	53.8	>100
4k	0	2,4-F_2	27.4	>100
4l	0	4-CF_3	92.5	>100
5a	2	H	>100	>100
5b	2	4-Cl	>100	>100
5c	2	4-Br	>100	>100

Table 2. In vitro enzyme inhibition by (*E*)-styryl acetoxyphenylsulfides and sulfones



Compound	*n*	X	IC_{50} (μM)	
			COX-2	COX-1
7a	0	H	>100	>100
7b	0	4-Cl	>100	>100
7c	0	4-Br	>100	>100
8a	2	H	>100	>100
8b	2	4-Cl	>100	>100
8c	2	4-Br	>100	>100
8d	2	2,4,6-$(OCH_3)_3$	>100	>100

To study the structure–activity relationship of these molecules (**4**), we have made (*Z*)-styryl acetoxyphenyl sulfones (**5**), (*E*)-styryl acetoxyphenyl sulfide (**7**), and (*E*)-styryl acetoxyphenyl sulfones (**8**) by different methods (Schemes 1–3). Analysis of COX-2 enzyme inhibition data clearly shows that either oxidation of the thio group or the change in the configuration of the molecule from (*Z*) to (*E*) results in the loss of activity (Table 2). The oxidation of (*Z*)-styryl acetoxyphenyl sulfides (**4**) to sulfones (**5**) could make the aromatic ring more electron deficient and consequently may hinder the transfer of the acetyl group to the serine residue or the molecule may not fit in the fatty acid binding site because of the bulkier sulfone group. Another interesting aspect of the structure–activity relationship of the sulfides (**4**) is their geometrical configuration. Because of the presence of a double bond in the molecule, styryl acetoxyphenyl sulfides exist as *trans* (*E*) or *cis* (*Z*) isomers. Spatial arrangement of atoms in a molecule are critical for drug ligand interactions, which always determines the ability of a molecule to bind to its receptor. Some of the drugs, which are potent inhibitors of various targets, exist as chiral molecules.[28] Drugs such as desloratadine (Clarinex®), fexofenadine (Allegra®), esomeprazole (Nexium®), etc. are more active as an (*R*)- or (*S*)-isomer as compared to the corresponding racemates. This suggests that geometrical and stereoisomers differ in their drug property and the spatial distribution of atoms in a molecule is critical for the biological activity of the drug.

Our study shows that (*Z*)-styryl acetoxyphenyl sulfides are more potent inhibitors of COX-2 enzymatic activity than the corresponding (*E*)-isomers, which are inactive. Styryl acetoxyphenyl sulfides in (*Z*)-configuration may be a perfect fit for the fatty acid binding pocket of COX-2, whereas molecules with (*E*)-configuration that are more spatially distributed than (*Z*)-isomers may not fit into the active site.

Studies involving selective covalent modification of serine (Ser^{516}) in COX-2 and interaction of (*Z*)-styryl

acetoxyphenyl sulfide (**4a**) with other residues in the catalytic domain are in progress.

4. Conclusions

In this report, we have synthesized a series of novel (*Z*)- and (*E*)-styryl acetoxyphenyl sulfides and sulfones and examined their activity against COX-1 and COX-2 enzymes. Our results show that only the (*Z*)-styryl acetoxyphenyl sulfides selectively inhibit COX-2. Unlike celecoxib, rofecoxib, and valdecoxib, these molecules are likely to inhibit COX-2 enzyme by covalently modifying the serine residue (Ser^{516}) at the active site. The structure–activity relationship of styryl acetoxyphenyl sulfides and sulfones demonstrates that (*Z*)-configuration and the unoxidized thio group are critical for the COX-2 inhibitory activity. This work led to the discovery of a series of compounds that are mechanistically similar to aspirin but possessing higher degree of selectivity towards COX-2 enzyme inhibition unlike aspirin.

5. Experimental

5.1. COX-inhibition-EIA assay

Cyclooxygenase activity of ovine COX-1 and COX-2 was assayed using COX inhibitory screening assay kit (Cayman Chemicals, MI). This assay directly measures $PGF_{2\alpha}$, that was produced by stannous chloride reduction of COX derived PGH_2 by enzyme immunoassay (EIA). This assay is more accurate and reliable than the peroxidase inhibition assay as shown by Gierse et al.[25] All assays were conducted in duplicate and IC_{50} values are the average of duplicate determinations for each compound. In brief, for the inhibition assay, hematin reconstituted purified COX-1 and COX-2 enzymes (six units) in a reaction buffer containing Tris–HCl (0.1 M, pH 8.0), 5 mM EDTA, 2 mM phenol, were pre-incubated at room temperature for 1 h with inhibitor concentrations ranging from 0.001 to 100 μM in DMSO followed by the addition of arachidonic acid (100 μM) for 2 min at 37 °C. Reactions were terminated by adding 50 μL of 1 M HCl followed by the addition of 100 μL of saturated stannous chloride. The final product $PGF_{2\alpha}$ formed was measured by EIA and IC_{50} values were determined following the instructions given in the kit manual.

5.2. Chemistry

5.2.1. General information. Melting points were determined in open capillary tubes using a Mel-Temp® electro thermal apparatus and are uncorrected. Proton NMR (^{1}H NMR) and carbon NMR (^{13}C NMR) spectra were determined in $CDCl_3$ or DMSO-d_6 solution on a Bruker Avance 400 spectrometer. Proton chemical shifts (δ) are expressed in parts per million (ppm) relative to tetramethylsilane as an internal standard. Spin multiplicities are given as s (singlet), d (doublet), br s (broad singlet), m (multiplet), and q (quartet). Coupling constants (*J*) are given in hertz (Hz). Elemental analyses were obtained by Quantitative Technologies Inc. (White House, New Jersey) and the results were within 0.4% of the calculated values unless otherwise mentioned.

5.2.2. Materials and methods. Reagents and solvents were purchased from common suppliers and were used without further purification. Reactions were monitored by thin-layer chromatography (TLC) on silica gel plates (60 Å; Aldrich), visualized under 254 nm ultraviolet light or iodine spray. Column chromatography separations were performed using silica gel (70–230 mesh) obtained from the Aldrich Company. The solvents used for elution varied depending on the compound and included either one or a combination of the following: petroleum ether, ethylacetate, chloroform, and methanol. All reactions were conducted under a nitrogen atmosphere unless otherwise noted. Yields were of purified product and were not optimized. Celecoxib was prepared according to the literature procedure.[6] Ethynylbenzenes were either purchased or prepared according to the procedure in the literature.[19]

5.2.3. General procedure for the synthesis of (*Z*)-styryl-2-hydroxyphenyl sulfides (3). To a stirred solution of sodium hydroxide (2 mmol) in absolute methanol (20 mL) was added dropwise 2-hydroxythiophenol (1 mmol) over a period of 0.75 h. On completion of the addition and when the reaction was no longer exothermic, phenylacetylene (1 mmol) was added, and the reaction mixture was refluxed for 24 h. The reaction mixture was then poured into ice-cold hydrochloric acid (10 mL) solution and was stirred for 10 min. The solution was extracted with ethyl acetate (2 × 50 mL). The combined organic layer was collected, washed with water (2 × 30 mL), dried over anhydrous Na_2SO_4, and concentrated in vacuo, to afford the respective (*Z*)-styryl hydroxyphenyl sulfides. Products **3** were used without further purification for the preparation of compounds **4**.

5.2.3.1. (*Z*)-Styryl-2-hydroxyphenyl sulfide (3a). This compound was obtained as light yellow solid (78%) by reaction of 2-hydroxythiophenol with phenylacetylene. Mp 71–73 °C; ^{1}H NMR ($CDCl_3$): δ 6.03 (d, J = 10.6 Hz, 1H), 6.15 (br s, OH), 6.52 (d, J = 10.6 Hz, 1H), 6.86–7.51 (m, 9H). Anal. Calcd for $C_{14}H_{12}OS$: C, 73.65; H, 5.30. Found: C, 73.95; H, 5.29.

5.2.3.2. (*Z*)-4-Fluorostyryl-2-hydroxyphenyl sulfide (3b). This compound was obtained as light yellow solid (81%) by reaction of 2-hydroxythiophenol with 4-fluorophenylacetylene. Mp 55–57 °C; ^{1}H NMR ($CDCl_3$): δ 6.08 (d, J = 10.5 Hz, 1H), 6.18 (br s, OH), 6.57 (d, J = 10.5 Hz, 1H), 6.91–7.58 (m, 8H). Anal. Calcd for $C_{14}H_{11}FOS$: C, 68.27; H, 4.50. Found: C, 68.05; H, 4.53.

5.2.3.3. (*Z*)-2-Fluorostyryl-2-hydroxyphenyl sulfide (3c). This product was obtained as a colorless liquid (72% yield) by reaction of 2-hydroxythiophenol with 2-fluorophenylacetylene. ^{1}H NMR ($CDCl_3$): δ 6.12 (d, J = 10.7 Hz, 1H), 6.23 (br s, OH), 6.68 (d, J = 10.6 Hz, 1H), 7.01–7.90 (m, 8H). Anal. Calcd for $C_{14}H_{11}FOS$: C, 68.27; H, 4.50. Found: C, 68.29; H, 4.52.

5.2.3.4. (*Z*)-4-Chlorostyryl-2-hydroxyphenyl sulfide (3d). This product was obtained as a light yellow crystalline solid (78% yield) by reaction of 2-hydroxythiophenol with 4-chlorophenylacetylene. Mp 51–53 °C; ^{1}H NMR ($CDCl_3$): δ 6.06 (d, J = 10.7 Hz, 1H), 6.16 (br s, OH), 6.46 (d, J = 10.7 Hz, 1H), 6.87–7.47 (m, 8H). Anal. Calcd for $C_{14}H_{11}ClOS$: C, 63.99; H, 4.22. Found: C, 64.20; H, 4.21.

5.2.3.5. (*Z*)-4-Bromostyryl-2-hydroxyphenyl sulfide (3e). This product was obtained as a light yellow crystalline solid (85% yield) by reaction of 2-hydroxythiophenol with 4-bromophenylacetylene. Mp 54–57 °C; ^{1}H NMR ($CDCl_3$): δ 6.07 (d, J = 10.5 Hz, 1H), 6.21 (br s, OH), 6.52 (d, J = 10.6 Hz, 1H), 6.99–7.60 (m, 8H). Anal. Calcd for $C_{14}H_{11}BrOS$: C, 54.73; H, 3.61. Found: C, 54.58; H, 3.63.

5.2.3.6. (*Z*)-4-Methylstyryl-2-hydroxyphenyl sulfide (3f). This product was obtained as a colorless liquid (77% yield) by reaction of 2-hydroxythiophenol with 4-methylphenylacetylene. ^{1}H NMR ($CDCl_3$): δ 2.30 (s, 3H), 5.98 (d, J = 10.6 Hz, 1H), 6.21 (br s, OH), 6.52 (d, J = 10.6 Hz, 1H), 6.97–7.50 (m, 8H). Anal. Calcd for $C_{15}H_{14}OS$: C, 74.34; H, 5.82. Found: C, 74.56; H, 5.85.

5.2.3.7. (*Z*)-4-Ethylstyryl-2-hydroxyphenyl sulfides (3g). This product was obtained as a colorless liquid (69% yield) by reaction of 2-hydroxythiophenol with 4-ethylphenylacetylene. ^{1}H NMR ($CDCl_3$): δ 1.24 (t, 3H), 2.62 (q, 2H), 6.02 (d, J = 10.5 Hz, 1H), 6.18 (br s, OH), 6.50 (d, J = 10.6 Hz, 1H), 7.02–7.57 (m, 8H). Anal. Calcd for $C_{16}H_{16}OS$: C, 74.96; H, 6.29. Found: C, 74.79; H, 6.31.

5.2.3.8. (*Z*)-4-Pentylstyryl-2-hydroxyphenyl sulfide (3h). This product was obtained as a colorless liquid (64% yield) by reaction of 2-hydroxythiophenol with 4-pentylphenylacetylene. ^{1}H NMR ($CDCl_3$): δ 0.86 (t, 3H), 1.28 (m, 4H), 1.45 (m, 2H), 2.55 (t, 2H), 5.97 (d, J = 10.6 Hz, 1H), 6.23 (br s, OH), 6.59 (d, J = 10.6 Hz, 1H), 6.86–7.48 (m, 8H). Anal. Calcd for $C_{19}H_{22}OS$: C, 76.47; H, 7.43. Found: C, 76.62; H, 7.39.

5.2.3.9. (*Z*)-3-Hydroxystyryl-2-hydroxyphenyl sulfide (3i). This product was obtained as a colorless liquid (66% yield) by reaction of 2-hydroxythiophenol with 3-hydroxyphenylacetylene. ^{1}H NMR ($CDCl_3$): δ 6.24 (br s, OH), 7.08–7.59 (m, 10H), 8.0 (br s, OH). Anal. Calcd for $C_{14}H_{12}O_2S$: C, 68.83; H, 4.95. Found: C, 68.51; H, 4.96.

5.2.3.10. (*Z*)-4-Methoxystyryl-2-acetoxyphenyl sulfide (3j). This product was obtained as a colorless liquid (74% yield) by reaction of 2-hydroxythiophenol with 4-methoxyphenylacetylene. ^{1}H NMR ($CDCl_3$): δ 3.82 (s, 3H, OCH_3), 6.11 (d, J = 10.5 Hz, 1H), 6.21 (br s, OH), 6.58 (d, J = 10.5 Hz, 1H), 6.91–7.55 (m, 8H). Anal. Calcd for $C_{15}H_{14}O_2S$: C, 69.74; H, 5.46. Found: C, 69.87; H, 5.48.

5.2.3.11. (*Z*)-2,4-Difluorostyryl-2-acetoxyphenyl sulfide (3k). This product was obtained as a colorless liquid (69% yield) by reaction of 2-hydroxythiophenol with 2,4-difluorophenylacetylene; ^{1}H NMR ($CDCl_3$: δ 6.26 (br s, OH), 6.35 (d, J = 10.5 Hz, 1H), 6.60 (d, J = 10.6 Hz, 1H), 6.80–7.79 (m, 7H). Anal. Calcd for $C_{14}H_{10}F_2OS$: C, 63.62; H, 3.81. Found: C, 63.41; H, 3.82.

5.2.3.12. (*Z*)-4-Trifluoromethylstyryl-2-acetoxyphenyl sulfide (3l). This product was obtained as a colorless semisolid (75% yield) by reaction of 2-hydroxythiophenol with 4-trifluorophenylacetylene. ^{1}H NMR ($CDCl_3$): δ 6.16 (br s, OH), 6.28 (d, J = 10.6 Hz,1H), 6.52 (d, J = 10.6 Hz, 1H), 7.14–7.78 (m, 8H). Anal. Calcd for $C_{15}H_{11}F_3OS$: C, 60.80; H, 3.74. Found: C, 60.58; H, 3.72.

5.2.4. General procedure for the synthesis of (*Z*)-styryl-2-acetoxyphenyl sulfides (4). A reaction mixture containing (*Z*)-styryl hydroxyphenyl sulfide (3 mmol), dry pyridine (3.2 mmol), and acetic anhydride (3.2 mmol) in 5 mL dry methylene chloride was stirred at room temperature for 6 h. Water was added to the reaction mixture, and the aqueous solution was extracted with methylene chloride (2×10 mL). The combined organic phase was washed with water, dried (Na_2SO_4), and filtered, and the solvent was concentrated in vacuo. Chromatography on silica gel (EtOAc/hexanes, 4:96) gave the desired product **4**.

5.2.4.1. (*Z*)-Styryl-2-acetoxyphenyl sulfide (4a). This product was obtained as a light yellow crystalline solid (59% yield) by reaction of acetylation with **3a**. Mp 54–56 °C; ^{1}H NMR ($CDCl_3$): δ 2.31 (s, 3H, $COCH_3$), 6.34 (d, J = 10.5 Hz,1H), 6.62 (d, J = 10.5 Hz, 1H), 7.12–7.57 (m, 9H); ^{13}C NMR ($CDCl_3$): δ 21.24 (CH_3), 122.37 (CH=CH), 128.58 (CH=CH), 123.27–150.31 (Ar–C), 169.62 ($OCOCH_3$). Anal. Calcd for $C_{16}H_{14}O_2S$: C, 71.08; H, 5.22. Found: C, 71.30; H, 5.23.

5.2.4.2. (*Z*)-4-Fluorostyryl-2-acetoxyphenyl sulfide (4b). This product was obtained as a yellow crystalline solid (65% yield) by reaction of acetylation with **3b**. Mp 42–44 °C; ^{1}H NMR ($CDCl_3$): δ 2.26 (s, 3H, $COCH_3$), 6.28 (d, J = 10.5 Hz, 1H), 6.58 (d, J = 10.5 Hz, 1H), 7.10–7.59 (m, 8H); ^{13}C NMR ($CDCl_3$): δ 21.20 ($COCH_3$),123.40 (CH=CH), 128.36 (CH=CH), 115.64–150.27 (Ar–C), 169.55 ($OCOCH_3$). Anal. Calcd for $C_{16}H_{13}FO_2S$: C, 66.65; H, 4.54. Found: C, 66.87; H, 4.53.

5.2.4.3. (*Z*)-2-Fluorostyryl-2-acetoxyphenyl sulfide (4c). This product was obtained as a colorless liquid (62% yield) by reaction of acetylation with **3c**. ^{1}H NMR ($CDCl_3$): δ 2.30 (s, 3H, $COCH_3$), 6.46 (d, J = 12.0 Hz, 1H), 6.78 (d, J = 10.0 Hz, 1H), 7.06–7.90 (m, 8H); ^{13}C NMR ($CDCl_3$): δ 21.18 ($COCH_3$), 123.59 (CH=CH), 128.95 (CH=CH), 115.63–150.37 (Ar–C), 169.57 ($OCOCH_3$). Anal. Calcd for $C_{16}H_{13}FO_2S$: C, 66.65; H, 4.54. Found: C, 66.68; H, 4.55.

5.2.4.4. (*Z*)-4-Chlorostyryl-2-acetoxyphenyl sulfide (4d). This product was obtained as a light yellow crystalline solid (68% yield) by reaction of acetylation with **3d**. Mp 36–38 °C; ^{1}H NMR ($CDCl_3$): δ 2.31 (s, 3H, $COCH_3$), 6.36 (d, J = 10.5 Hz, 1H), 6.55 (d, J = 10.5 Hz, 1H), 7.12–7.56 (m, 8H); ^{13}C NMR ($CDCl_3$): δ 21.22 (CH_3), 123.57 (CH=CH), 128.96 (CH=CH), 126.17–150.32 (Ar–C), 169.55 ($OCOCH_3$). Anal. Calcd for $C_{16}H_{13}ClO_2S$: C, 63.05; H, 4.30. Found: C, 62.91; H, 4.32.

5.2.4.5. (*Z*)-4-Bromostyryl-2-acetoxyphenyl sulfide (4e). This product was obtained as a light yellow crystalline solid (65% yield) by reaction of acetylation with **3e**. Mp 40–42 °C; ^{1}H NMR ($CDCl_3$): δ 2.31 (s, 3H, $COCH_3$), 6.37 (d, J = 10.5 Hz, 1H), 6.52 (d, J = 10.0 Hz, 1H), 7.09–7.60 (m, 8H); ^{13}C NMR ($CDCl_3$): δ 22.74 ($COCH_3$), 125.08 (CH=CH), 128.89 (CH=CH), 123.00–151.83 (Ar–C), 171.06 ($OCOCH_3$). Anal. Calcd for $C_{16}H_{13}BrO_2S$: C, 55.03; H, 3.75. Found: C, 54.83; H, 3.76.

5.2.4.6. (*Z*)-4-Methylstyryl-2-acetoxyphenyl sulfide (4f). This product was obtained as a colorless liquid (67% yield) by reaction of acetylation with **3f**. ^{1}H NMR ($CDCl_3$): δ 2.30 (s, 3H, $COCH_3$), 2.33 (s, 3H), 6.18 (d, J = 10.5 Hz, 1H), 6.56 (d, J = 10.5 Hz, 1H), 7.07–7.59 (m, 8H). Anal. Calcd for $C_{17}H_{15}O_2S$: C, 72.06; H, 5.34. Found: C, 71.79; H, 5.37.

5.2.4.7. (*Z*)-4-Ethylstyryl-2-acetoxyphenyl sulfide (4g). This product was obtained as a colorless liquid (59% yield) by reaction of acetylation with **3g**. ^{1}H NMR ($CDCl_3$): δ 1.27 (t, 3H), 2.31 (s, 3H, $COCH_3$), 2.66 (q, 2H), 6.15 (d, J = 10.5 Hz, 1H), 6.53 (d, J = 10.6 Hz, 1H), 7.02–7.67 (m, 8H). Anal. Calcd for $C_{18}H_{17}O_2S$: C, 72.70; H, 5.76. Found: C, 73.01; H, 5.77.

5.2.4.8. (*Z*)-4-Pentylstyryl-2-acetoxyphenyl sulfide (4h). This product was obtained as a colorless liquid (54% yield) by reaction of acetylation with **3h**. ^{1}H NMR ($CDCl_3$): δ 0.93 (t, 3H), 1.34 (m, 4H), 1.76 (m, 2H), 2.29 (s, 3H,$COCH_3$), 2.69 (t, 2H), 6.12 (d, J = 10.5 Hz, 1H), 6.59 (d, J = 10.6 Hz, 1H), 7.11–7.61 (m, 8H). Anal. Calcd for $C_{21}H_{24}O_2S$: C, 74.08; H, 7.10. Found: C, 74.30; H, 7.13.

5.2.4.9. (*Z*)-3-Acetoxystyryl-2-acetoxyphenyl sulfide (4i). A reaction mixture containing 3-hydroxystyryl-2-hydroxyphenyl sulfide (3 mmol), dry pyridine (6.4 mmol), and acetic anhydride (6.4 mmol) in 5 mL dry methylene chloride was stirred at room temperature for 6 h. Water was added to the reaction mixture, and the aqueous solution was extracted with methylene chloride (2×10 mL). The combined organic phase was washed with water, dried (Na_2SO_4), and filtered, and the solvent was concentrated in vacuo. Chromatography on silica gel (EtOAc/hexanes, 4:96) gave as a colorless liquid (63% yield). ^{1}H NMR ($CDCl_3$): δ 2.28 (s, 3H, $COCH_3$), 2.41 (s, 3H, $COCH_3$), 7.08–7.59 (m, 10H); ^{13}C NMR ($CDCl_3$): δ 21.14 (CH_3), 30.55 (CH_3), 123.69 (CH=CH), 128.46 (CH=CH), 121.77–151.68 (Ar–C), 169.32 ($COCH_3$), 173.52 ($COCH_3$); Anal. Calcd for $C_{17}H_{16}O_4S$: C, 64.54; H, 5.10. Found: C, 64.69; H, 5.07.

5.2.4.10. (*Z*)-4-Methoxystyryl-2-acetoxyphenyl sulfide (4j). This product was obtained as a colorless liquid (64% yield) by reaction of acetylation with **3j**. ^{1}H NMR ($CDCl_3$): δ 2.31 (s, 3H, $COCH_3$), 3.82 (s, 3H, OCH_3), 6.21 (d, J = 10.5 Hz, 1H), 6.58 (d, J = 10.5 Hz, 1H), 6.84–7.55 (m, 8H); ^{13}C NMR ($CDCl_3$): δ 21.22 (CH_3), 55.45 (OCH_3), 123.45 (CH=CH), 128.67 (CH=CH), 122.42–155.23 (Ar–C), 169.60 ($OCOCH_3$). Anal. Calcd for $C_{17}H_{15}O_3S$: C, 68.20; H, 5.05. Found: C, 67.93; H, 5.06.

5.2.4.11. (*Z*)-2,4-Difluorostyryl-2-acetoxyphenyl sulfide (4k). This product was obtained as a colorless liquid (65% yield) by reaction of acetylation with **3l**. ^{1}H NMR ($CDCl_3$): δ 2.34 (s, 3H, $COCH_3$), 6.45 (d, J = 10.5 Hz, 1H), 6.70 (d, J = 10.5 Hz, 1H), 6.80–7.79 (m, 7H); ^{13}C NMR ($CDCl_3$): δ 21.18 ($COCH_3$), 123.22 (CH=CH), 128.67 (CH=CH), 120.76–150.30 (Ar–C), 169.53 ($COCH_3$). Anal. Calcd for $C_{16}H_{12}F_2O_2S$: C, 62.73; H, 3.95. Found: C, 62.74; H, 3.95.

5.2.4.12. (*Z*)-4-Trifluoromethylstyryl-2-acetoxyphenyl sulfide (4l). This product was obtained as a colorless semisolid (55% yield) by reaction of acetylation with **3m**. ^{1}H NMR ($CDCl_3$): δ 2.32 (s, 3H, $COCH_3$), 6.48 (d, J = 10.5 Hz,1H), 6.60 (d, J = 10.5 Hz, 1H), 7.14–7.78 (m, 8H); ^{13}C NMR ($CDCl_3$): δ 21.14 ($COCH_3$), 123.65 (CH=CH), 128.88 (CH=CH), 120.55–150.44 (Ar–C), 169.58 ($OCOCH_3$). Anal. Calcd for $C_{17}H_{12}F_3O_2S$: C, 60.53; H, 3.58. Found: C, 60.69; H, 3.59.

5.2.5. General procedure for the synthesis of (*Z*)-styryl-2-acetoxyphenyl sulfone (5). To a solution containing (*Z*)-styryl-2-acetoxyphenyl sulfides (15 mmol) in 20 mL of glacial acetic acid was added 30% hydrogen peroxide (8 mL) dropwise at room temperature. After the addition was complete, the reaction mixture was allowed to stirr at room temperature for 16 h. The mixture was then poured into ice-cold water and stirred for 10 min. The separated solid filtered, and dried to give required compound **5**.

5.2.5.1. (*Z*)-Styryl-2-acetoxyphenyl sulfone (5a). This product was obtained as a white solid (88% yield) by oxidation of **4a**. Mp 98–100 °C; ^{1}H NMR ($CDCl_3$): δ 2.09 (s, 3H, $COCH_3$), 6.40 (d, J = 10.6 Hz, 1H), 6.94 (d, J = 10.6 Hz, 1H), 6.96–7.70 (m, 9H). Anal. Calcd for $C_{16}H_{14}O_4S$: C, 63.56; H, 4.67. Found: C, 63.59; H, 4.64.

5.2.5.2. (*Z*)-4-Chlorostyryl-2-acetoxyphenyl sulfone (5b). This product was obtained as a white solid (81% yield) by oxidation of **4d**. Mp 84–86 °C; ^{1}H NMR ($CDCl_3$): δ 2.21 (s, 3H, $COCH_3$), 6.42 (d, J = 10.5 Hz, 1H), 6.58 (d, J = 10.5 Hz, 1H), 7.09–7.92 (m, 8H). Anal. Calcd for $C_{16}H_{13}ClO_4S$: C, 57.06; H, 3.89. Found: C, 57.19; H, 3.87.

5.2.5.3. (*Z*)-4-Bromostyryl-2-acetoxyphenyl sulfone (5c). This product was obtained as a white solid (86% yield) by oxidation of **4e**. Mp 88–90 °C; ^{1}H NMR ($CDCl_3$): δ 2.22 (s, 3H, $COCH_3$), 6.46 (d, J = 10.5 Hz, 1H), 6.62 (d, J = 10.5 Hz, 1H), 7.01–7.98 (m, 8H). Anal. Calcd for $C_{16}H_{13}BrO_4S$: C, 50.41; H, 3.44. Found: C, 50.53; H, 3.45.

5.2.6. General procedure for the synthesis of (*E*)-styryl-2-hydroxyphenyl sulfides (6). To a solution containing phenylacetylene (15 mmol) in 20 mL of glacial acetic acid was added manganese(III) acetate dihydrate (7.5 mmol) at room temperature. The mixture was stirred and heated in an oil bath, and then 2-hydroxy thiophenol (22.5 mmol) was added just before refluxing. The dark brown color of manganese(III) acetate dihydrate disappeared within 2 min. The solvent was removed in vacuo and the residue was triturated with water followed by extraction with chloroform. The extract was dried over anhydrous Na_2SO_4, filtered, and concentrated to dryness. The products were separated by silica gel column chromatography using chloroform as the eluting solvent, resulted the desired product (**6**).

5.2.6.1. (*E*)-Styryl-2-hydroxyphenyl sulfide (6a). This product was obtained as a solid (88% yield) by reaction of 2-hydroxythio phenol with phenylacetylene. Mp 92–94 °C; ^{1}H NMR ($CDCl_3$): δ 6.71 (d, J = 15.3 Hz, 1H), 7.42 (d, J = 15.3 Hz, 1H), 6.61–7.82 (m, 9H), 8.16 (br s, OH). Anal. Calcd for $C_{14}H_{12}OS$: C, 73.65; H, 5.30. Found: C, 73.79; H, 5.31.

5.2.6.2. (*E*)-4-Chlorostyryl-2-hydroxyphenyl sulfide (6b). This product was obtained as a white solid (82% yield) by reaction of 2-hydroxythio phenol with 4-chlorophenyl acetylene. Mp 96–98 °C; ^{1}H NMR ($CDCl_3$): δ 6.86 (d, J = 15.3 Hz, 1H), 7.52 (d, J = 15.3 Hz, 1H), 6.93–7.91 (m, 8H), 8.21 (br s, OH). Anal. Calcd for $C_{14}H_{11}ClOS$: C, 63.99; H, 4.22. Found: C, 64.07; H, 4.23.

5.2.6.3. (*E*)-4-Bromostyryl-2-hydroxyphenyl sulfide (6c). This product was obtained as a white solid (81% yield) by reaction of acetylation with **6c**. Mp 102–104 °C; ^{1}H NMR ($CDCl_3$): δ 6.94 (d, J = 15.4 Hz, 1H), 7.60 (d, J = 15.0 Hz, 1H), 7.13–8.00 (m, 8H), 8.18 (br s, OH). Anal. Calcd for $C_{14}H_{11}BrOS$: C, 54.73; H, 3.61. Found: C, 54.69; H, 3.61.

5.2.7. General procedure for the synthesis of (*E*)-styryl-2-acetoxyphenyl sulfides (7). A reaction mixture containing styryl hydroxyphenyl sulfide (3 mmol), dry pyridine (3.2 mmol), and acetic anhydride (3.2 mmol) in 5 mL dry methylene chloride was stirred at room temperature for 6 h. Water was added to the reaction mixture, and the aqueous solution was extracted with methylene chloride (2×10 mL). The combined organic phase was washed with water, dried (Na_2SO_4), and filtered, and the solvent was concentrated in vacuo. Chromatography on silica gel (EtOAc/hexanes, 4:96) gave the desired acetate **7**.

5.2.7.1. (*E*)-Styryl-2-acetoxyphenyl sulfide (7a). This product was obtained as a solid (78% yield) by reaction of acetylation with **6a**. Mp 77–79 °C; ^{1}H NMR ($CDCl_3$): δ 2.29 (s, 3H, $COCH_3$), 6.79 (d, J = 15.3 Hz, 1H), 7.49 (d, J = 15.3 Hz, 1H), 6.69–7.87 (m, 9H). Anal. Calcd for $C_{16}H_{14}O_2S$: C, 71.08; H, 5.22. Found: C, 70.92; H, 5.20.

5.2.7.2. (*E*)-4-Chlorostyryl-2-acetoxyphenyl sulfide (7b). This product was obtained as a white solid (82% yield) by reaction of acetylation with **6b**. Mp 86–88 °C; ^{1}H NMR ($CDCl_3$): δ 2.35 (s, 3H, $COCH_3$), 6.93 (d, J = 15.3 Hz, 1H), 7.62 (d, J = 15.3 Hz, 1H), 7.03–8.01 (m, 8H). Anal. Calcd for $C_{16}H_{13}ClO_2S$: C, 63.05; H, 4.30. Found: C, 63.09; H, 4.28.

5.2.7.3. (*E*)-4-Bromostyryl-2-acetoxyphenyl sulfide (7c). This product was obtained as a white solid (81% yield) by reaction of acetylation with **6c**. Mp 92–94 °C; ^{1}H NMR ($CDCl_3$): δ 2.39 (s, 3H, $COCH_3$), 6.94 (d, J = 15.4 Hz, 1H), 7.60 (d, J = 15.0 Hz, 1H), 7.13–8.00 (m, 8H). Anal. Calcd for $C_{16}H_{13}BrO_2S$: C, 55.03; H, 3.75. Found: C, 55.26; H, 3.74.

5.2.8. General procedure for the synthesis of (*E*)-styryl-2-acetoxyphenyl sulfones (8). To a solution containing (*E*)-styryl-2-acetoxyphenyl sulfides (15 mmol) in 20 mL of glacial acetic acid was added 30% hydrogen peroxide (8 mL) dropwise at room temperature. After the addition was complete, the reaction mixture was allowed to stir at room temperature for 16 h. The mixture was then poured into ice-cold water and stirred for 10 min. The separated solid filtered, and dried to give required compound **8**.

5.2.8.1. (*E*)-Styryl-2-acetoxyphenyl sulfone (8a). This product was obtained as a solid (81% yield) by oxidation of **7a**. Mp 114–116 °C; ^{1}H NMR ($CDCl_3$): δ 1.97 (s, 3H, $COCH_3$), 6.83 (d, J = 15.4 Hz, 1H), 7.59 (d, J = 15.4 Hz, 1H), 6.79–7.97 (m, 9H). Anal. Calcd for $C_{16}H_{14}O_4S$: C, 63.56; H, 4.67. Found: C, 63.82; H, 4.66.

5.2.8.2. (*E*)-4-Chlorostyryl-2-acetoxyphenyl sulfone (8b). This product was obtained as a white solid (86% yield) by oxidation of **7b**. Mp 120–122 °C; ^{1}H NMR ($CDCl_3$): δ 2.35 (s, 3H, $COCH_3$), 6.93 (d, J = 15.4 Hz, 1H), 7.62 (d, J = 15.4 Hz, 1H), 7.03–8.06 (m, 8H). Anal. Calcd for $C_{16}H_{13}ClO_4S$: C, 57.06; H, 3.89. Found: C, 56.96; H, 3.90.

5.2.8.3. (*E*)-4-Bromostyryl-2-acetoxyphenyl sulfone (8c). This product was obtained as a white solid (85% yield) by oxidation of **7c**. Mp 138–140 °C; ^{1}H NMR ($CDCl_3$): δ 2.39 (s, 3H, $COCH_3$), 6.97 (d, J = 15.5 Hz, 1H), 7.63 (d, J = 15.0 Hz, 1H), 7.23–8.07 (m, 8H). Anal. Calcd for $C_{16}H_{13}BrO_4S$: C, 50.41; H, 3.44. Found: C, 50.47; H, 3.43.

5.2.9. General procedure for the synthesis of 2-hydroxy phenylthio acetic acid (9). To a stirred solution of sodium hydroxide (12.8 g, 320 mmol) in absolute methanol (200 mL) was added dropwise 2-hydroxythio phenol

(20 g, 160 mmol) over a period of 0.75 h. On completion of the addition and when no longer the reaction was exothermic, chloroacetic acid (18.14 g, 192 mmol) was added portion wise, and the reaction mixture was refluxed for 5 h. This mixture was then poured into ice-cold hydrochloric acid solution (20 mL) and was stirred for 10 min. The solution was extracted with ethyl acetate (2×100 mL). The combined organic layer was collected, washed with water (2×50 mL), dried over anhydrous Na_2SO_4, and concentrated under vacuo, to afford as a oil in 98.4% yield. ^{1}H NMR ($CDCl_3$): δ 3.46 (s, 2H, CH_2), 6.79–7.46 (m, 4H), 8.41 (br s, OH). Anal. Calcd for $C_8H_8O_3S$: C, 52.16; H, 4.38. Found: C, 52.29; H, 4.36. Product **9** was used immediately without further purification for the preparation of compound **10**.

5.2.10. General procedure for the synthesis of 2-hydroxyphenyl sulfonyl acetic acid (10). To a solution containing 2-hydroxyphenylthio acetic acid (15 g, 82 mmol) in 75 mL of glacial acetic acid was added 30% hydrogen peroxide (30 mL) dropwise at room temperature. After the addition was complete, the reaction was stirred for 16 h at room temperature. The solution was concentrated in vacuo, and the residue was filtered, washed with petroleum ether and dried to give a white powder. Yield 65%; Mp 125–126 °C; ^{1}H NMR ($CDCl_3$): δ 3.86 (s, 2H, CH_2), 6.89–7.56 (m, 4H), 8.52 (br s, OH). Anal. Calcd for $C_8H_8O_5S$: C, 44.44; H, 3.73. Found: C, 44.36; H, 3.74. Product **10** was used without further purification for the preparation of compound **11**.

5.2.11. General procedure for the synthesis of (*E*)-styryl-2-hydroxyphenyl sulfone (11). A mixture of 2-hydroxyphenyl sulfonyl acetic acid (**10**) (4.6 mmol), aromatic aldehydes (5.1 mmol), benzoic acid (0.7 mmol), and piperidine (0.6 mmol) in toluene (20 mL) was refluxed for 4 h with continuous removal of water using a Dean–Stark water separator. The reaction mixture was cooled to room temperature, diluted with ethyl acetate, washed with saturated $NaHCO_3$ solution (20 mL), water (30 mL), and the organic phase was separated, and dried (Na_2SO_4). The solvent was removed in vacuo. Chromatography on silica gel (chloroform) gave the desired product **11**.

5.2.11.1. (*E*)-Styryl-2-hydroxyphenyl sulfone (11a). This product was obtained as a white solid (56% yield) by reaction of **10** with benzaldehyde. Mp126–128 °C; ^{1}H NMR ($CDCl_3$): δ 6.78 (d, J = 15.2 Hz, 1H), 6.91–7.77 (m, 8H), 7.52 (d, J = 15.4 Hz, 1H), 8.92 (br s, OH).

5.2.11.2. (*E*)-4-Chlorostyryl-2-hydroxyphenyl sulfone (11b). This product was obtained as a white solid (63% yield) by reaction of **10** with 4-chlorobenzaldehyde. Mp 132–134 °C; ^{1}H NMR ($CDCl_3$): δ 6.88 (d, J = 15.0 Hz,1H), 6.95–7.87 (m, 8H), 7.62 (d, J = 15.5 Hz, 1H), 8.97 (br s, OH).

5.2.11.3. (*E*)-4-Bromostyryl-2-hydroxyphenyl sulfone (11c). This product was obtained as a white solid (59% yield) by reaction of **10** with 4-bromobenzaldehyde. Mp 145–147 °C; ^{1}H NMR ($CDCl_3$): δ 6.82 (d, J = 15.4 Hz, 1H), 6.95–7.61 (m, 8H), 7.52 (d, J = 15.4 Hz, 1H), 8.86 (br s, OH).

5.2.11.4. (*E*)-2,4,6-Trimethoxystyryl-2-hydroxyphenyl sulfone (11d). This product was obtained as a white solid (63% yield) by reaction of **10** with 2,4-6-trimethoxybenzaldehyde. Mp 132–134 °C; ^{1}H NMR ($CDCl_3$): δ 3.89 (s, 9H, OCH_3), 6.02 (s, 2H, Ar–H), 6.82 (d, J = 15.4 Hz, 1H), 6.95–7.61 (m, 6H), 7.52 (d, J = 15.4 Hz,1H), 8.78 (br s, OH).

5.2.12. General procedure for the synthesis of (*E*)-styryl-2-acetoxyphenyl sulfone (8). A reaction mixture containing (*E*)-styryl hydroxyphenyl sulfone (3 mmol), dry pyridine (3.2 mmol), and acetic anhydride (3.2 mmol) in 5 mL dry methylene chloride was stirred at room temperature for 6 h. Water was added to the reaction mixture, and the aqueous solution was extracted with methylene chloride (2×10 mL). The combined organic phase was washed with water, dried (Na_2SO_4), and filtered, and the solvent was concentrated in vacuo. Chromatography on silica gel (EtOAc/hexanes, 4:96) gave the desired acetate **8**.

5.2.12.1. (*E*)-Styryl-2-acetoxyphenyl sulfone (8a). This product was obtained as a solid (81% yield) by reaction of acetylation with **11a**. Mp 114–116 °C; ^{1}H NMR ($CDCl_3$): δ 1.97 (s, 3H, $COCH_3$), 6.83 (d, J = 15.4 Hz, 1H), 6.79–7.97 (m, 9H), 7.59 (d, J = 15.4 Hz, 1H).

5.2.12.2. (*E*)-4-Chlorostyryl-2-acetoxyphenyl sulfone (8b). This product was obtained as a white solid (86% yield) by reaction of acetylation with **11b**. Mp 120–122 °C; ^{1}H NMR ($CDCl_3$): δ 2.35 (s, 3H, $COCH_3$), 6.93 (d, J = 15.4 Hz, 1H), 7.03–8.06 (m, 8H), 7.62 (d, J = 15.4 Hz, 1H).

5.2.12.3. (*E*)-4-Bromostyryl-2-acetoxyphenyl sulfone (8c). This product was obtained as a white solid (85% yield) by reaction of acetylation with **11c**. Mp 138–140 °C; ^{1}H NMR ($CDCl_3$): δ 2.39 (s, 3H, $COCH_3$), 6.97 (d, J = 15.5 Hz, 1H), 7.23–8.07 (m, 8H), 7.63 (d, J = 15.0 Hz, 1H).

5.2.12.4. (*E*)-2,4,6-Trimethoxystyryl-2-acetoxyphenyl sulfone (8d). This product was obtained as a white solid (83% yield) by reaction of acetylation with **11d**. Mp 122–124 °C; ^{1}H NMR ($CDCl_3$): δ 2.34 (s, 3H, $COCH_3$), 3.89 (s, 9H, OCH_3), 6.02 (s, 2H, Ar–H), 6.82 (d, J = 15.4 Hz, 1H), 6.95–7.61 (m, 6H), 7.52 (d, J = 15.4 Hz, 1H).

Acknowledgements

This work was supported by grants from the Department of Defense, US Army Breast Cancer Research Program, DAMD 17-02-1-0579, Onconova Therapeutics, Inc. New Jersey and Fels Foundation, Philadelphia.

References and notes

1. Hamberg, M.; Samuelsson, B. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 899.
2. Fu, J. Y.; Masferrer, J. L.; Seibert, K.; Raz, A.; Needleman, P. *J. Biol. Chem.* **1990**, *265*, 16737.
3. (a) Smith, W. L.; DeWitt, D. L. *Adv. Immunol.* **1996**, *62*, 167; (b) Herschman, H. R. *Biochem. Biophys. Acta* **1996**, *125*, 1299.
4. Meade, E. A.; Smith, W. L.; DeWitt, D. L. *J. Biol. Chem.* **1993**, *268*, 6610.
5. Allison, M. C.; Howatson, A. G.; Torrance, C. J.; Lee, F. D.; Russell, R. I. G. *N. Engl. J. Med.* **1992**, *327*, 749.
6. Penning, T. D.; Tally, J. J.; Bertenshaw, S. R.; Carter, J. S.; Collins, P. W.; Docter, S.; Graneto, M. J.; Lee, L. F.; Malecha, J. W.; Miyashiro, J. M.; Rogers, R. S.; Rogier, D. J.; Yu, S. S.; Anderson, G. D.; Burton, E. I. G.; Cogburn, J. N.; Gregory, S. A.; Koboldt, C. M.; Perkins, W. E.; Seibert, K.; Veenhuizen, A. W.; Zhang, Y. Y.; Isakson, P. C. *J. Med. Chem.* **1977**, *40*, 1347.
7. Prasit, P.; Wang, Z.; Brideau, C.; Chan, C. C.; Charleson, S.; Cromlish, W.; Ethier, D.; Evans, J. F.; Ford-Hutchinson, A. W.; Gauthier, J. Y. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1773.
8. Nicoll-Griffith, D. A.; Yergey, J. A.; Trimble, L. A.; Silva, J. M.; Li, C.; Chauret, N.; Gauthier, J. Y.; Grimm, E.; Leger, S.; Roy, P.; Therien, M.; Wang, Z.; Prasit, P.; Zamboni, R.; Young, R. N.; Brideau, C.; Chan, C. C.; Mancini, J.; Riendeau, D. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2683.
9. Gierse, J. K.; McDonald, J. J.; Hauser, S. D.; Rangwala, S. H.; Koboldt, C. M.; Seibert, K. *J. Biol. Chem.* **1996**, *271*, 15810.
10. Fabiola, G. F.; Pattabhi, V.; Nagarajan, K. *Bioorg. Med. Chem.* **1998**, *6*, 2337.
11. Rahim, M. A.; Rao, P. N. P.; Knaus, E. E. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2753.
12. Roth, G. J.; Stanford, N.; Majerus, P. W. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 3073.
13. Vane, J. R. *Nat. New Biol.* **1971**, *231*, 232.
14. Smith, J. B.; Willis, A. L. *Nature* **1971**, *231*, 235.
15. Cheng, Y.; Austin, S. C.; Rocca, B.; Koller, B. H.; Coffman, T. M.; Grosser, T.; Lawson, J. A.; FitzGerald, G. A. *Science* **2002**, *296*, 539.
16. Vane, J. R. *Science* **2002**, *296*, 474.
17. Kalgutkar, A. S.; Crews, B. C.; Rowlinson, S. W.; Garner, C.; Seibert, K.; Marnett, L. J. *Science* **1998**, *280*, 1268.
18. Kalgutkar, A. S.; Kozak, K. R.; Crews, B. C.; Hochgesang, G. P.; Marnett, L. J. *J. Med. Chem.* **1998**, *41*, 4800.
19. Janine, C. *Ann. Chim.* **1948**, *12*, 393.
20. Reddy, M. V. R.; Reddy, S. *Synthesis* **1983**, 322.
21. Reddy, M. V. R.; Reddy, S. *Acta. Chim. Hung.* **1984**, *115*, 269.
22. Nguyen, V.; Nishino, H.; Kajikawa, S.; Kurosawa, K. *Tetrahedron* **1998**, *54*, 11445.
23. Reddy, M. V. R.; Vijayalakshmi, S.; Reddy, D. B.; Reddy, P. V. R. *Phosphorus, Sulfur, Silicon* **1991**, *60*, 209.
24. Reddy, D. B.; Reddy, N. S.; Reddy, S.; Reddy, M. V. R.; Balasubramanyam, S. *Org. Prep. Proc. Int.* **1988**, *20*, 205.
25. Gierse, J. K.; Koboldt, C. M.; Walker, M. C.; Seibert, K.; Isakson, P. C. *Biochem. J.* **1999**, *339*, 607.
26. (a) Wells, I.; Marnettt, L. J. *Biochemistry* **1992**, *31*, 9520; (b) Picot, D.; Loll, P. J.; Garavito, R. M. *Nature* **1994**, *367*, 243; (c) Loll, P. J.; Picot, D.; Garavito, R. M. *Nat. Struct. Biol.* **1995**, *2*, 637.
27. Vane, J. R.; Botting, R. M. *Thromb. Res.* **2003**, *110*, 255.
28. Triggle, D. J. *Drug Discovery Today* **1997**, *2*, 138.